EFFECTS OF MULTIPLE EXOGENOUS ENZYME PRODUCTS ON *IN VITRO* FERMENTATION BY RUMEN MICROBES IN BATCH AND CONTINUOUS CULTURE FERMENTATION

A THESIS
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

TRENT DADO

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

MARSHALL STERN, ALFREDO DICOSTANZO

AUGUST 2019

Acknowledgements

Thank you to my family for the love and support throughout my education. Work hard. Get smart.

Thank you to my wife, Mickie, for reviewing documents and listening to my enthusiastic, science rants.

Thank you to my advisers for allowing me to explore my passions and supplying endless guidance in my graduate experience.

Thank you to my fellow students for the great conversations and memories made during my tenure.

Thank you to all my friends for supplying good times and a break from reality.

ABSTRACT

Research has demonstrated that supplementing exogenous enzymes to ruminants has potential to improve feed digestion and animal performance. Enzyme products with fibrolytic, proteolytic and amylolytic activities and diets with diverse composition have been used to test enzyme efficacy. Responses to these conditions have been variable. A series of *in vitro* experiments were designed to 1) screen enzyme products in ruminal batch culture to determine effects on digestibility and gas production and 2) further examine effective enzymes in dual-flow continuous culture to determine their effect on microbial fermentation. In Exp. 1, seven treatments, including a multi-enzyme blend (MEB), ferulic acid esterase (FAE), protease (PRO), α-amylase (AAM), β-glucanase (BGL), xylanase (XYL) or control (CON), were added to each of 3 diets with forage:concentrate ratios of 50:50 (Diet 1), 30:70 (Diet 2) or 10:90 (Diet 3), at 6 dosage rates (0, 125, 250, 500, 1000 and 10,000 mg of enzyme/kg of diet DM) in batch culture. Dose was removed from analysis and all doses were combined and examined together. In vitro total dry matter digestibility (IVTDMD) was greater ($P < 0.05$) for BGL compared with all other treatments in Diet 1 and Diet 2. In Diet 3, BGL was greater ($P < 0.05$) than AAM and CON. Control and XYL had greater ($P < 0.05$) total gas production than MEB, FAE, and AAM. Rate of gas production (mL/h/g of DM) was also affected ($P < 0.05$) by diet and enzyme for the first 24 h of fermentation with CON having the fastest rate at 3 h, generally. Overall, BGL increased digestibility without generating as much gas as CON. In Exp. 2, BGL and PRO from Exp. 1, a cellulase (CEL) preparation and control (CON) were examined in eight dual-flow continuous culture over 3 periods. The diet was a 40:60 (forage:concentrate) and enzyme was supplied at 1000 mg/kg of diet DM. Digestibility of apparent DM and OM tended to be greater ($P < 0.10$) for CON compared with CEL.

Volatile fatty acid (VFA) concentration and nitrogen metabolism were not affected ($P > 0.05$) by enzyme treatment. In summary, the increase in DM and OM digestibility found with BGL and other effects of enzymes on fermentation in batch culture were not observed in dual-flow continuous culture.

Table of Contents

List of Tables v
List of Figures vi
Literature Review 1
INTRODUCTION 1
ACTIVITIES OF ENZYMES 2
EXOGENOUS ENZYME SUPPLEMENTATION RESPONSES 7
SUMMARY 20
Effects of multiple exogenous enzyme products on in vitro fermentation by rumen microbes in batch and continuous culture fermenters 21
INTRODUCTION 22
MATERIALS AND METHODS 24
RESULTS AND DISCUSSION 32
CONCLUSION 42
Bibliography 58

List of Tables

Table 1. Ingredient and chemical composition of Exp. 1 diets. 43
Table 2. Ingredient and chemical composition of Exp. 2 diet. 44
Table 3. Diet composition of donor steers. 45
Table 4. Effects of exogenous enzyme supplementation on DM, OM and fiber digestion in continuous culture 46
Table 5. Effect of exogenous enzyme supplementation on VFA concentration in continuous culture. 47
Table 6. Effect of exogenous enzyme supplementation on nitrogen metabolism in continuous culture. 48

List of Figures

Figure 1. Effect of enzyme supplementation on diets with varying forage:concentrate on DMD in batch culture. 49
Figure 2. Effect of enzyme supplementation on diets with varying forage:concentrate on IVTDMD in batch culture. 50
Figure 3. Effect of diet on total gas production in batch culture. 51
Figure 4. Effect of exogenous enzyme on total gas production in batch culture. 52
Figure 5. Effect of exogenous enzyme supplementation on rate of gas production in Diet 1 53
Figure 6. Effect of exogenous enzyme supplementation on rate of gas production in Diet 2 54
Figure 7. Effect of exogenous enzyme supplementation on rate of gas production in Diet 3 55
Figure 8. Effect of enzyme supplementation on diets with varying forage:concentrate on PF in batch culture. 56
Figure 9. Effects of exogenous enzyme supplementation on pH in continuous culture... 57

Literature Review

INTRODUCTION

Humans have long been dependent on ruminant species for meat, milk, labor and fiber. Ruminants serve a valuable role in human society as a unique adaptation allows them to ferment cellulosic plant materials undegradable by mammalian tissue. Ruminant domestication is estimated to have occurred over 10,000 years ago (Cole and Ronning, 1974). Their domestication occurred alongside the initiation of crop cultivation as ruminants could provide draft capabilities without competing for human food sources. As vital as ruminants were for early man, ruminants fit a specialized, necessary role in today's agriculture system. Ruminants convert vast renewable resources from rangeland, crop-residues and other by-products into more valuable and palatable food sources (Oltjen and Beckett, 1996).

Ruminant animals depend on a synergistic relationship with a diverse microbial ecosystem within the reticulo-rumen for pregastric fermentation of feed. These microbes produce enzymes that degrade cellulosic plant material. Anaerobic microbial fermentation yields a variety of end-products. Fermentation produces volatile fatty acids (VFA) which are absorbed through the rumen epithelium and undergo lipogenesis and gluconeogenesis to provide as much as of 74% of the metabolizable energy for the host animal (Siciliano-Jones and Murphy, 1988). Growth and outflow of microbial populations from the rumen serve as a vital amino acid source for the host animal (Clark et al., 1992). Rumen microbes can also utilize non-protein nitrogen (NPN) such as urea or nucleic acids to synthesize protein. In addition, the host animal can recycle ammonia

across the rumen epithelium and incorporate it into saliva as urea. This allows microbes to convert recycled NPN into microbial crude protein. This synergistic relationship is not 100% efficient. Microbial fermentation generates gaseous products: primarily methane and CO_2. Production and eructation of methane represents a significant loss of ingested energy in cattle (Johnson and Johnson, 1995).

The production cycle of ruminants presents a multitude of opportunities to increase the production efficiency of ruminants. The challenge for farmers and animal scientists is to increase production to supply a growing demand for milk and meat while minimizing effects of ruminant production on the environment. These tasks must be accomplished in an economically favorable fashion. A key to accomplishing these goals is to modulate rumen fermentation. To effectively change rumen fermentation, it is crucial to understand rumen ecology and function. Typical rumen function is dependent on microbial attachment and enzymatic degradation of substrate. This function can be modified by altering microbial species or their metabolism resulting in downstream effects on ruminant performance. For several decades, use of exogenous feed enzymes has been a source of interest to modify rumen fermentation and ruminant production efficiency. This review will outline current knowledge as it applies to enzymatic breakdown of feed and application of exogenous enzymes in ruminant production.

ACTIVITIES OF ENZYMES

Fibrolytic Enzymes

Fibrolytic enzymes are characterized by their ability to digest portions of the plant cell wall. Cellulose and hemicellulose are major structural polysaccharides present in plant cell walls (Van Soest, 1994). Enzymes responsible for conversion of cellulose and

hemicelluslose to soluble sugars are referred to as cellulases and hemicellulases (Bhat and Hazlewood, 2001).

Cellulose is a linear polymer of glucose subunits which are linked by β-1,4 glycosidic bonds. Each glucose unit in the polymer is rotated 180° from the previous glucose unit in the chain with repeating units referred to as cellobiose. Chain length of cellulose varies between 100 and 14,000 glucose units (Beguin and Aubert, 1994). Hydrogen bonds hold these glycan chains together to form complex and random miofibril arrangements within the primary plant cell wall (Albersheim, 1975). Cellulose matrixes in plant cell walls may be either crystalline or amorphous. When cellulose chains are highly ordered and contain an abundance of hydrogen bonds, they form crystalline structures. Conversely, loosely bound cellulose chains form amorphous regions. Native cell walls differ significantly in their degree of crystallinity (Bhat and Hazlewood, 2001). Due to the complex structure of cellulose, multiple specific enzymes contribute to cellulase activity. Cellulose hydrolyzation is completed by three classes of enzymes; endocellulases, exocellulases and β-glucosidases. Endocelluases, including endoglucanase, endo-β-1,4-glucanase, carboxymethyl cellulase and β-1,4-glucan glucanohydrolase, are responsible for hydrolyzing cellulose at random locations in the chain to create oligomers of cellulose. Exocellulases, including exoglucanase, exo-β-1,4-glucanase and cellulose β-1,4-cellobiosidase, hydrolyze non-reducing ends of cellulose chains to produce cellobiose. Finally, β-glucosidase, including cellobiase and glucohyrdolase, release glucose from cellobiose and hydrolyze cello-oligosaccharides (Bhat and Hazzlewood, 2001).

Cellulose miofibrils are linked to hemicellulosic polysaccharides. Hemicellulose is composed of shorter-chain polysaccharides and is the primary polysaccharide outside of cellulose in plant cell walls (Fan et al., 1981). Hemicellulose, unlike the well-defined structure of cellulose, is more difficult to characterize. Traditionally, hemicelluloses are polysaccharides which remain after hot acid and chelator treatment and are extracted by alkaline treatment. These remaining polysaccharides have dramatically different structures and physiochemical properties (Scheller and Ulvskov, 2010). Due to this heterogeneity, hemicellulose can be described by different sugar residues they contain. Van Soest (1994) described hemicellulose as a linear xylan core polymer consisting of β-1,4 linking xylose residues. Cheng et al. (1991) described hemicellulose similarly but indicated that xylan backbones may be substituted or linked with other sugar residues such as arabinose and glucuronic acid. Scheller and Ulvskov (2010) described hemicellulose as a cell wall polysaccharide that is neither cellulose or pectin and has β-1,4-linked backbone of glucose, xylose or mannose. Finally, Bhat and Hazlewood (2001) stated hemicellulose may be denoted by the main sugar residue present in the polymer such that hemicelluloses can be called xylans, glucomannans, galatans or arabinans. The most common being xylans and glucomannans.

Due to the complexity of biochemical structure and composition of natural hemicelluloses, enzymes needed to degrade this fraction of plant cell walls must be equally diverse. To degrade xylan cores, or hemicellulose, both xylanases and β-1,4 xylosidase are needed (Bhat and Hazlewood, 2001). Xylanases cleave β-1,4 linkages at random with unsubstituted regions of the polymer preferred (Tenkanen et al., 2013). Xylosidase hydrolyzes xylooligosaccharides to xylose (Biely, 1985). Multiple other

enzymes are involved in digestion of hemicellulolytic side chains. These include, but are not limited to, β-monnosidase, α-D-glucouronidase, α-D-glactosidase, actyl-xylan esterases and ferulic acid esterase (White et al., 1993).

To add another layer of complexity, lignin and phenolic acids, limit plant cell wall digestion. Cross linking of lignin with other cell wall polysaccharides with ferulic acid bridges leads to this limitation (Jung and Allen, 1995). Lignin is the most difficult plant cell wall component to degrade. Lignins are complex polymers resulting from oxidative coupling of 4-hydroxyphenylpropanoids. Their complexity is due to a high degree of randomness in linkage generation within the polymer. Astounding numbers of different isomers are created and make it improbable that any two lignin macromolecules are identical (Ralph et al., 2004). Linkages between plant cell wall polysaccharide and lignins and between two lignin chains are hydrolyzed by a variety of esterases including carboxylesterases, acetlyesterases and pectin methylesterase.

Proteolytic Enzymes

Use of exogenous proteolytic enzymes had been ignored because of the assumption they would lead to increased proteolytic degradation in the rumen and lead to inefficient use of nitrogen (Eun and Beauchemin, 2005). However, McAllister et al. (1993) suggested protein in endosperm of cereal grains may be a limiting factor in digestion of starch. Moreover, cell wall protein residues such as tyrosine and cysteine may create bridges between cell wall polysaccharides and lignins (Bacic et al., 1988). Rumen proteolysis compared with carbohydrate fermentation is complicated by the presence of microbial protein and other endogenous protein sources (Van Der Walt and Meyer, 1988). Protein degradation in the rumen encompasses multiple steps including

solubilization, extracellular hydrolysis, transport, deamination and formation of end-products. Hydrolysis of protein in the rumen is accomplished by use of amino acid arylamidase, trypsin, carboxypeptidase and chymotrypsin, which have been found to be active in rumen fluid of cows fed a variety of diets (Prins et al., 1983). Breakdown of peptides to amino acids is done by peptidases. In the rumen, the majority of peptidase activity is aminopeptidase (Wallace, 1996).

Bacteria, protozoa and fungi exhibit proteolytic activity (Selinger et al., 1996). *Provotella ruminicola* has been identified as a predominant proteolytic bacterial species (Wallace and Brammall, 1985). Its cysteine protease activity is common in other bacterial species as well (Wallace, 1996). Ciliated protozoa, which do not hydrolyze soluble protein as readily as ruminal bacteria, also produce cysteine and aspartic proteases (Forsberg et al., 1984).

Amylolytic Enzymes

Cereal grains constitute a significant portion of diets used for intensive production of ruminant livestock in the United States. Starch content of common cereal grains range from 58 to 78 %DM (Herrera-Saldana et al., 1990). Starch is composed of two polymers, amylose and amylopectin. Amylose consists of α-1,4-linked glucose units and is 900 to 3000 residues in length (French, 1973). Most commercial starch sources contain from 0 to 20% amylose (Rooney and Pflugfelder, 1986). Amylopectin is a larger and more branched-chain polymer. It contains α-1,4-linked glucose units joint by α-1,6 bonds (Kotarski et al., 1992). Several abundant species of bacteria in the rumen produce amylolytic enzymes. Amylolyitc enzymes include α-amylase, maltohexaohydrolase, maltotetrahydrolase, β-amylase, α-glucosidase, glucoamylase, pullulanase, and

isoamylase. These enzymes can hydrolyze, at least partially, amylopectin. All enzymes listed except pullulanase and isoamylase hydrolyze amylose. This is due to their specificity to hydrolyze endo-α-1,6 bonds found only in amylopectin (Kotarski et al., 1992). Protozoa also impact starch digestion by either ingesting bacteria in numbers that decrease ruminal fermentation rate of starch or ingesting starch granules and decreasing accessibility of starch to faster growing bacteria (Oxford, 1955; Eadie and Hobson, 1962).

EXOGENOUS ENZYME SUPPLEMENTATION RESPONSES

Literature cited in this section is divided based on the model that is used to determine the efficacy of enzymes. The first model is *in vivo* including both dairy and beef. The second model is *in vitro* including batch culture incubations and continuous culture fermentation.

In Vivo Models

Beef

In the 1960's, researchers began examining exogenous enzymes in growing and finishing beef cattle, dairy calves and heifers as well as *in vitro*. Burroughs et al. (1960) used a commercially-available dried enzyme product, Agrozyme, in fattening beef cattle. Agrozyme had amylolytic, proteolytic, and cellulolytic activity (Mcallister et al., 1999). They fed Agrozyme in 10 feeding experiments to a total of 325 cattle. Average daily gain (ADG) increased by 7% while feed-to-gain ratio was reduced 6%. Differences in gain and feed conversion were observed without any significant differences in dry matter (DM), protein or cellulose digestibility (Burroughs et al., 1960). Agrozyme, along with

three other commercials enzymes, Zymo-Papst, Rhozyme and Takamine were fed at different inclusion levels with or without diethylstilbestrol (DES). When all enzymes were fed with DES in a corn-alfalfa hay ration, they observed 0.28 kg increase in ADG over control steers and 0.13 kg over steers given only DES (Nelson and Damon, 1960). Rovics and Ely (1962) conducted two experiments using a bacterial-fungal enzyme. In the experiment, 44 steers fed a silage-dense ration were divided in two equal groups and supplemented with 0 or 0.005 kg of an enzyme preparation. In the second experiment, 50 heifers were fed a high corn and low roughage diet and divided into equal groups and again fed either 0 or 0.005 kg of enzyme. Steers and heifers fed the enzyme gained 0.04 and 0.05 kg more per head per d, respectively, when compared with the control (Rovics and Ely, 1962). However, positive results were not consistently found in other research. Clark et al. (1961) fed two different preparations of Rhozyme, which had primarily amylolytic and proteolytic activity, to fattening Herefords and no significant differences in ADG were observed. Other researchers found enzymes to decrease beef performance. Perry et al. (1960) used a factorial design to determine the effect of high moisture or shelled corn and three commercial enzyme supplements on steer performance. They found a significant reduction in ADG with Agrozyme treatment when compared with the control. Steers fed the other two enzymes treatments were not statistically different from the control steers (Perry et al., 1960).

These early studies established potential benefits of enzyme supplements to beef cattle. However, research failed to elaborate on the effects of diet composition, type of enzyme activity and level of enzyme application (McAllister, 2000). More recent work has focused on these factors in an aim to clarify effects of exogenous enzymes in beef

cattle. A variety of diets have been explored. Beauchemin et al. (1995) fed 72 steers either alfalfa hay, cubed timothy hay or barley silage. Diets were supplemented with incremental concentrations of cellulase and xylanase. Moderate inclusion of enzyme increased ADG by 30% in the alfalfa hay diet while high concentrations of enzyme addition increased ADG by 36% in timothy hay. No response was observed at any enzyme inclusion when barley silage was fed to steers. Similar effects were observed by Mcallister et al. (1999) with steers fed a backgrounding diet containing 82.5% barley silage followed by a finishing diet with 70% barley-ryegrass silage and 30% barley grain. Diets were supplemented with two commercial fungal enzymes preparations (Finnfeeds International Ltd., Malborough, UK) with cellulase and xylanase activity. Enzymes increased ADG from d 0 to 56 and increased final weight at 120 d in steers fed the backgrounding diet. In the finishing phase, ADG was greater for steers given the enzyme supplement. Beauchemin et al. (1995) attributed increases in weight gain to an improvement in forage digestibility. This was supported by Feng et al. (1996) who used *in situ*, *in vitro* and *in vivo* techniques to determine the effect of cellulase and xylanase enzyme preparations on fiber digestibility. They found *in situ* NDF disappearance, total tract DM and NDF digestibility to be greater for diets supplied with enzymes before feeding.

Contrary to logic, fibrolytic enzymes applied to high-grain, finishing diets had more consistent results than high-forage diets. Beauchemin et al. (1997) fed high concentrate diets (95.1% on a DM basis) consisting of either corn and corn silage or barley and barley silage to finishing steers. Concentrate was treated with either one of two enzyme mixtures of different cellulase and xylanase activities. The enzyme with

greater xylanase activity increased feed conversion of steers fed the barley diet by 11%. Enzyme treatment did not affect performance of steers fed the corn-based diet. In a separate study, heifers fed a barley-based, high-concentrate diet (92.2% on a DM basis) were supplemented with a cellulase and xylanase enzyme mixture. Heifers fed enzyme had a 9% increase in ADG without an increase in DMI (Beauchemin et al., 1999a).

Various enzyme activities and dosage rates have been examined in beef diets in various studies. A meta-analysis compiled by Tirado-González et al. (2018) included 45 experiments. When fibrolytic enzymes were used in diets with less than 50% forage, ADG increased by 0.30 kg/d. In experiments that used fibrolytic enzymes in higher forage diets observed an improvement in DMI but no increase was seen in ADG. Enzymes with proteolytic and amylolytic activity have also been studied in beef animals, but to a lesser extent than fibrolytic enzymes. Vera et al. (2012) found no change in steer growth performance in the growing phase when diets containing 30% dried distillers grains with solubles (DDGS) were supplemented with proteolytic enzymes in. However, in the finishing phase ADG tended to be greater in supplemented steers versus control steers. An *Aspergillus oryzae* extract with α-amylase activity was used in combination with a variety of diets to determine possible interactions between enzyme supplementation, forage source, corn processing methods and DMI. Extract was found to increase ADG in steers fed cottonseed hulls as the forage source in finishing diets. *A. oryzae* extract supplementation also increased ADG of heifers in the first 28 d on feed regardless of corn processing method (Tricarico et al., 2007). DiLorenzo et al. (2011) used an α-amylase supplement (Rumistar; DSM Nutritional Products, Inc., Kaiseraugst,

Switzerland) in a finishing diet using either steam-flaked corn or dry rolled corn. The enzyme supplement did not affect nutrient digestibility or the growth of steers.

Several attempts have been made to identify appropriate application rates of enzyme products. This effort is difficult because dosage may depend on diet and physiological state of the animal (Beauchemin et al., 2003). Vargas et al. (2013) fed a high grain diet were dosed with 0, 2, 4 or 6 mg/kg of diet DM of Fibrozyme (Alltech Inc., Nicholasville, KY, USA) to crossbred Zebu and Brown Swiss steers. No change was seen in performance of steers at any enzyme concentration. Beauchemin et al. (1995) also used multiple dosage rates of a fibrolytic enzyme. They noted that in some diets lower doses were more effective in increasing ADG than high doses.

Dairy

Enzymes in dairy nutrition were first applied to dairy calf diets as early as 1951. Williams and Knodt (1951) added either papain or pancreatin powder to milk replacer. Calves fed papain grew faster than those fed pancreatin, but the authors noted all calves had poor growth rates. Further work with milk replacers demonstrated negative effects of feeding enzymes to dairy calves. Fries et al. (1958) pre-digested a plant-based milk replacer with malt diastase and papain and fed it to dairy calves for 60 d. No improvement was found with any treatment. Finally, multiple amylase enzymes were studied in milk replacers with corn or other starch sources. Blood sugar concentrations were measured in calves after feeding. When fungal amylase was added to the milk replacer, no change was observed in blood sugar concentration (Okamoto et al., 1959). Moreover, when carbohydrates were added to milk replacers with or without amylolytic enzymes, calves grew faster with the enzyme compared with no enzyme addition.

However, calves fed replacer without carbohydrate grew faster than both groups fed replacer with carbohydrates (Netke et al., 1960). Because young calves have a primarily monogastric digestive system, effects of enzymes in their diet may not be applicable to mature dairy cows with functional ruminant stomachs.

Research of exogenous enzymes on lactating cow performance did not commence until the mid-1990's (McAllister, 2000). Similar to enzyme use in beef production, enzyme supplementation in dairy has yielded variable results. Discrepancies between experimental results have been attributed to a variety of factors including type and activity of the enzyme, rate of supplementation, method of providing the enzyme to animal and composition of the diet (Beauchemin et al., 2004). A recent meta-analysis evaluated similar heterogeneity sources including experiment duration, type and application rate of enzyme, form (liquid or solid) and method of enzyme application (Arriola et al., 2017).

Fibrolytic, proteolytic, and amylolytic enzymes have been used in experiments with lactating dairy cows with cellulases and xylanases being the most common (Meale et al., 2014). Ortiz-Rodea et al. (2013) examined effects of various enzymes on dairy cow performance through meta-analytical techniques. They analyzed observations from 29 experiments which included 52 treatments and 9 different types of enzymes. Addition of any type of exogenous enzyme had no effect on milk yield or milk fat, lactose and protein content (Ortiz-Rodea et al., 2013). Interestingly, when an enzyme mixture containing cellulase, xylanase, α-amylase and protease activities was fed to Brown Swiss cows, increases in fermentation and production measurements were observed. Dry matter intake and digestibility of all major nutrient classes improved with enzyme

supplementation. Possibly leading to significant increase in milk yield and milk protein content with cows fed the supplement (Gado et al., 2009).

Physiologic state of animals may play a role in effectiveness of enzyme supplementation because the response to enzymes is hypothesized to increase during times of compromised fiber digestion and energy limitation (Beauchemin et al. 2003). Thus, numerous experiments examined effects of enzymes in early lactation of dairy cows. DeFrain et al. (2005) determined the effect of feeding α-amylase to Holstein cows during the transition period. After measuring metabolic indicators, they found that α-amylase supplementation increased plasma glucose. There was no increase in β-hydroxybutyrate and nonesterified fatty acids in postpartum cows supplemented with enzyme, indicating a shift in metabolism from lipids to carbohydrates (DeFrain et al., 2005). In a similar fashion, cows in early lactation (46 ± 10 d in milk) were fed a diet containing 0, 0.5 or 1.0 mL enzyme/kg of diet DM. Adding fibrolytic enzyme did not affect milk yield but decreased DMI of cows fed the higher dose of enzyme. Therefore, efficiency of milk production linearly increased with increasing enzyme addition (Holtshausen et al., 2011). Rode et al. (1999) fed a diet supplemented with a cellulase and xylanase mixture to dairy cows. The experiment began at parturition and continued for 12 wk. Digestibility of NDF, ADF and crude protein were all increased with enzyme supplementation. Milk production also tended to increase with enzyme treatment. Peters et al. (2015) also used a cellulase and xylanase-based enzyme to determine effects of enzyme supplementation in early lactation, however, this experiment continued into mid lactation. No differences in digestibility of DM, crude protein, NDF or ADF were observed between control groups and cows supplemented with enzyme in either mid or

early lactation. Enzyme addition did not affect milk yield or milk components in mid and early lactation.

Method of enzyme application to cows' feed has also been explored. Yang et al. (2000) applied a fibrolytic enzyme to either a total mixed ration (TMR) or to a barley-based concentrate. Enzyme was dissolved and either sprayed onto the TMR before feeding or added to the concentrate portion of the ration before mixing. The ration was fed to Holstein cows in early lactation. Milk yield (kg/d) was higher for cows when enzyme was applied to the concentrate fraction (37.4) than cows on the control (35.3) or cows with enzyme applied to the total mixed ration (35.2). Bowman et al. (2002) compared application of fibrolytic enzymes to three different portions of a diet. Treatments included an enzyme applied to concentrate (45% of TMR), enzyme applied to the diet supplement (4% of TMR), and to the premix (0.2% of TMR). All treatments supplied 1.0 g of enzyme per cow per d. Digestibility of NDF and ADF was higher when enzyme was applied to the concentrate when compared with the control. When enzyme was applied to smaller portions of the diet, only numerical increases in digestibility were detected compared with the control. No difference in milk production and composition were observed among treatments. In a meta-analysis conducted by Arriola et al. (2017), effect of exogenous fibrolytic enzymes on cow performance was summarized over 17 experiments. The authors concluded their data did not support the notion that method of enzyme application affects performance of dairy cows.

In Vitro Models

Effect of exogenous enzyme supplementation on ruminant performance and digestion is variable. Many have used alternative *in vitro* methods to predict enzymes' *in*

vivo potential. With the objective to develop a rational selection assay or string of assays for exogenous enzymes for ruminants, Colombatto et al. (2003b) found that biochemical properties and hydrolytic capacities of enzymes alone could not predict the performance of the enzyme *in vitro*. Therefore, *in vitro* systems including batch culture incubations and continuous culture fermentations have been used to assess fiber degradability and fermentative effects of exogenous enzyme supplementation (Colombatto et al., 2003a,b)

Batch Culture

The *in vitro* fermentation methods described by Goering and Van Soest (1970) and Tilley and Terry (1963) are widely used to determine digestibility of forages. Procedures described by Tilley and Terry (1963) were designed to be reproducible and to handle many samples in a single experiment. They described a two-step procedure. First step being a 48 h fermentation of 0.5 g of forage with 10 mL of strained rumen fluid and 40 mL of a buffer solution. In the second step, the fermentation vessel was centrifuged and supernatant discarded. Residue was incubated in a pepsin solution for another 48 h. From these two steps, the apparent digestibility of the forage can be calculated (Tilley and Terry, 1963). Goering and Van Soest (1970), exposed fermented material to neutral detergent solution instead of exposing it to pepsin. In this way, they calculated true digestibility of forage. Exogenous enzymes are often evaluated using one of these two procedures or a slight modification of the procedures to determine digestibility of feed substrate. Researchers have examined a variety of enzymes, rates of application and feed substrates using batch culture techniques.

Many different enzyme products with a variety of enzyme activities have been used in *in vitro* batch cultures. Eun and Beauchemin (2007a) assessed 23 experimental enzyme products. Thirteen of the products contained endoglucanase activity and the remaining 10 enzymes contained xylanase activity. Alfalfa hay was inoculated with 10 mL of strained rumen fluid and 40 mL of buffer. Gas production was measured 4 times during 18 h of fermentation and apparent organic matter digestibility (OMD) was calculated at completion of fermentation. All but two and three endoglucanase enzymes increased gas production and OMD, respectively, compared with the control. Only half of the xylanase enzymes increased gas production and only two enzymes increased OMD. Based on these results, linear regression demonstrated a strong association between endoglucanase activity and OMD and no association between xylanase activity and OMD. In another experiment, (Eun and Beauchemin, 2007b), examined the efficacy of four enzyme products with multiple activities including endoglucanase, xylanase, exoglucanase and protease. Products were tested at varying doses with corn silage or alfalfa hay as substrate. Only endoglucanase and exoglucanase activity correlated with improvement of NDF digestibility of corn silage. Only endoglucanase activity tended correlate with improvement in NDF digestibility of alfalfa. A meta-analysis examined effects of fibrolytic enzymes on *in vitro* fiber digestibility (Tirado-Gonzalez et al., 2018). In high forage diets, cellulase enzymes were found to increase *in vitro* dry matter digestibility across all sources of rumen fluid (sheep, dairy and beef). In the same analysis, fibrolytic enzymes had a negative effect on *in vitro* NDF and ADF digestibility when evaluated in beef and dairy ruminal fluids (Tirado-Gonzalez et al., 2018).

Efficacy of amylolytic enzymes have been assessed in *in vitro* batch cultures. Klingerman et al. (2009) examined an enzyme product with primarily α-amylase activity in a 6 h batch culture. After VFA analysis, amylolytic enzyme linearly increased apparent total VFA production for both flint and dent but not for floury corn. Similarly, Rojo Rubio et al. (2001) used an α-amylase product from *Bacillus licheniformis* in an *in vitro* fermentation with either sorghum, steam-rolled sorghum or corn as the substrate. Enzyme supplement increased starch digestion across the three substrates.

Proteolytic enzymes have also been assessed for their potential use in ruminant diets through *in vitro* batch culture fermentation. Young et al. (2012) examined the effect of two protease enzymes on fermentation of corn silage during the ensiling process. Adding 2,000 mg/kg (wet-weight basis) to corn silage ensiled for 150 d increased *in vitro* starch digestibility. As part of a larger *in vivo* experiment, Eun and Beauchemin (2005) measured *in vitro* gas production and digestibility of various fractions of the TMR with proteolytic enzyme supplementation. Exogenous proteolytic enzyme enhanced forage and concentrate digestibility with a greater response found with barley-based grain.

Colombatto et al. (2003b) screened 25 enzymes with a variety of fibrolytic, amylolytic and proteolytic activities. Enzymes were added at a rate of 1.5 mg/kg DM of forage with corn silage or alfalfa hay used as substrates. After an 18-h batch culture digestion, apparent dry matter digestibility (DMD) was determined and enzymes were ranked based on their relative increase in DMD compared with a control. Using a stepwise multiple regression, a positive correlation between xylanase and protease activity and DMD of alfalfa hay was observed. However, a negative correlation was observed between corn silage and xylanase activity. Approximately one-third of *in vitro*

DMD was explained by enzyme activities. Based on these regressions, it is apparent unique and specific relationships exist between feedstuffs and enzyme activity.

Continuous-Culture Rumen Fermenters

Continuous-culture (CC) rumen fermenters, which are also used to model *in vivo* rumen fermentation can be used as an alternative to batch culture incubations. In contrast to batch culture technique, CC systems remove fermentation end-products, and maintain microbial fermentation for longer periods (Hristov et al., 2012). An early design of a CC system could maintain a rumen culture for up to 10 h (Adler et al., 1958). Stewart et al. (1961) renovated the system to encompass an inflow and outflow system controlled by electronic solenoids. This system maintained VFA and pH values similar to ruminal observations. However, mechanical malfunction limited fermentation to 24 h (Stewart et al. 1961). Slyter et al. (1964) simplified construction and operation of the system to sustain operation past 7 d. Volatile fatty acid and methane values in this system were similar to values obtained *in vivo* along with consistent fermentation patterns between 4 and 21 d of continuous culture (Slyter et al., 1964). Hoover et al. (1976) incorporated a dual effluent removal system for differential removal of solids and liquids. This system allowed for rapid input of buffer while allowing solid particles to remain longer within the vessel and subsequently allowed for increased protozoal numbers (Hoover et al. 1976). In modification of this system, Hannah et al. (1986) decreased fermenter volume, included a coaxial heat exchanger and continuously purged the vessel with N_2. Values for true OM, CP, and amino acid digestion in this system were similar to those obtained *in vivo* (Hannah et al., 1986).

Use of continuous culture systems to determine efficacy of exogenous enzyme on rumen fermentation has been limited. Two experiments examined effects of various pH levels with addition of exogenous enzymes. Yang et al. (2002) used a fibrolytic enzyme (Promote, Agribrands Inc., St. Louis, MO) in fermenters at pH levels 5.5, 6.0 or 6.5. Fermenters were supplied a diet containing 50% forage and 50% barley-based concentrate (DM basis). Increasing pH from 5.5 to 6.0 or 6.5 increased total VFA concentrations and degradability of DM, OM and fiber. Enzyme addition did not affect total VFA concentration but increased molar proportions of acetate and reduced that of propionate. In addition, NDF and ADF digestibility increased with enzyme supplementation compared with the control treatment. Crude protein and bacterial protein synthesis were not affected (Yang et al., 2002). Consistent with these observations, Colombatto et al. (2003a) used a CC system with two set pH ranges (5.4 to 6.0 and 6.0 to 6.7) to determine the influence of a proteolytic enzyme mixture on a TMR with fresh forages. Differences in pH ranges were established by changing the concentration of base in the artificial saliva. Degradability of OM, NDF and ADF were greater for fermenters kept at the higher pH range while protein degradation was not affected by pH. Addition of proteolytic enzyme increased NDF digestibility at both pH levels but to a greater extent in the high pH range. Total VFA production, protein degradation and microbial protein synthesis were not affected by enzyme addition. Vera et al. (2012) also used an exogenous proteolytic enzyme in CC. Proteolytic enzyme was added to a diet with or without dried distillers grains with solubles (DDGS). Total VFA concentration tended to increase with enzyme supplementation in both diets. Digestibility of DM, OM and NDF were increased with enzyme supplementation but only with DDGS.

SUMMARY

Enzymatic hydrolysis of feed in the rumen is a complex and intricate process. Many microbes and their enzymes are responsible for degradation of unique structures and chemical bonds which comprise feed. Ultimately, performance of ruminants depends heavily on hydrolysis of plant cell walls for production of VFA's and microbial protein. In an attempt to improve this fermentative process, researchers have explored the use of exogenous enzymes. Over decades of experimentation using many different models, effect of enzyme supplementation on ruminal fermentation or performance has been remarkably variable. Although sources of variation such as diet, enzyme activity, physiologic stage of animal, dose, and method of enzyme application have been identified, no pattern has been discovered to predict an enzymes efficacy in a given situation. Therefore, it is necessary to individually evaluate enzyme products. The objective remains to determine the mechanism of action of enzyme products so they may be properly administered to ruminants to improve rumen fermentation and ultimately animal performance.

Effects of multiple exogenous enzyme products on in vitro fermentation by rumen microbes in batch and continuous culture fermenters

T.S. Dado, M.D. Stern, A. DiCostanzo

INTRODUCTION

Exogenous feed enzyme use in ruminant diets has been examined thoroughly over the last decades and continues to invoke interest of researchers (Adesogan et al., 2014). Enzyme products are produced by either solid state or submerged liquid fermentation (Considine and Coughlan, 1989). A seed culture of fungal or bacterial species is grown in appropriate culture media and conditions for the specific microbe's optimal growth and production of enzyme (Lee et al., 1998). After fermentation, enzymes are separated from other fermentation end-products and the source microbe. Enzyme isolation from growth medium and microbes can be completed through homogenization or ultrasonication (Ravindran and Jaiswal, 2016). Direct application of commercially-available enzymes to the livestock industry is difficult because enzymes are developed for alternative industries including food, fuel, textile and chemical industries (Bhat and Hazlewood, 2001).

The goal of enzyme feed additives is to decrease cost of producing milk and meat from ruminant animals by increasing performance, decreasing inputs or both. This can be accomplished by increasing efficiency of digestion and metabolism of feed. Three general classes of enzyme activities have been explored in ruminant nutrition; fibrolytic, amylolytic and proteolytic (Sujani and Seresinhe, 2015). Whether fiber is from forages or grain, it comprises a large portion of ruminant diets and is the target of fibrolytic enzyme supplementation. Neutral detergent fiber (NDF) that is present in forage at a level of 30 to 70% on a DM basis is less than 50% digestible in the rumen (Van Soest, 1994). Improving forage NDF digestibility consequently led to greater DM intake (Dado and Allen, 1995), larger fat-corrected milk yield (Oba and Allen, 1999) and higher efficiency

of microbial protein production in beef steers and dairy cows (Firkins et al., 1986; Oba and Allen, 2000). Although counterintuitive, proteolytic enzymes have also been explored for their potential to increase fiber digestibility. It is hypothesized that exogenous proteolytic enzymes act by removing structural barriers that enhance microbial access to degradable fiber (Nsereko et al., 2000). Use of amylolytic enzymes is less common as ruminal digestion of starch is not considered limiting and rapid fermentation of excessive amounts of starch may lead to acidosis (Owens et al., 1996). However, it has been proposed that amylolytic enzymes may decrease variability seen in ruminal starch digestion (Firkins et al., 2001; Tricario et al., 2008).

Response to enzyme addition in ruminants has been variable. In beef, nutrient digestibility and average daily gain have been increased with enzyme addition (Balci et al., 2007; Gómez-Vázquez et al., 2011). Enzymes have also shown no effect on these characteristics (Krueger et al., 2008; Vera et al., 2012). Similar variability has been noted with enzyme supplementation in dairy diets. Milk production and nutrient digestibility increased through enzyme supplementation (Lopuszanka-Rusek and Bilik, 2011). In contrast, other studies (Holtshausen et al., 2011; Eun and Beauchemin, 2005) found no significant increase in milk production with enzyme addition. Variations in diet composition, enzyme activity, enzyme application level and method of enzyme application may affect the response to enzymes (Beauchemin et al., 2004).

Animal feeding studies can determine if enzyme products will enhance animal performance or feed utilization however *in vivo* studies are expensive and labor-intensive (Stern et al., 1997). Thus, *in vitro* screening techniques may assist in selecting appropriate enzyme candidates by accommodating large number of samples with high

precision (Beauchemin et al., 2004). With the objective to develop a feed enzyme to increase ruminant performance, the present study screened 6 commercial enzymes at 6 dosage levels on 3 diets with varying levels of concentrate. The products' efficacy was determined by measuring fiber digestibility and gas production using an *in vitro* batch culture. Three enzymes were subsequently selected to determine their effects on *in vitro* fermentation characteristics in continuous culture fermentation.

MATERIALS AND METHODS

Experimental Protocol

Two *in vitro* systems were used to evaluate various exogenous enzymes (EE) on fiber degradation and various other fermentation parameters. Experiment 1 utilized an *in vitro* batch culture system (Goering and Van Soest, 1970). The experiment was conducted as a completely randomized design with three replicates and a factorial arrangement of treatments; 6 EE × 6 dosage rates × 3 diets. Due to the size of the factorial, enzymes were randomly paired so the experiment was conducted in three separate subsequent assays. Diet substrate without enzyme (dosage rate 0) was used as a control (CON). Experiment 2 used eight dual-flow continuous culture fermenters (Hannah et al., 1986) including four treatments (two enzymes from Exp. 1, an additional enzyme product and control) with two replicates over three periods to generate six experimental units per treatment.

Enzyme Treatments

Enzyme products were assigned names according to the primary enzyme activity listed on the manufacturers' label or distinguished as an enzyme blend. All enzyme

products were supplied by PMI Nutrition (Arden Hills, MN) and were in granular or powder form.

The commercial enzyme products used in Exp. 1 were protease (PRO), α-amylase (AAM), β-glucanase (BGL), xylanase (XYL), ferulic acid esterase (FAE) and a multi-enzyme blend (MEB) developed by various manufacturers. Dilutions were calculated to achieve final dosage rates of 125, 250, 500, 1000 and 10,000 mg of enzyme/kg of diet DM. Enzymes were dissolved in incremental rates in 50 mL of distilled water so when 0.2 mL of the enzyme solution were added to the serum bottles, the desired dosage rate was achieved. Enzyme solution was added to the fermentation vessels at the time of inoculation.

BGL and PRO enzyme products from Exp. 1 and a cellulase (CEL) enzyme with previously shown *in vitro* efficacy (*Unpublished Results*) were used in Exp. 2. Enzymes were top-dressed onto the diet to supply 1000 mg/kg of diet DM/d and administered to the fermenter through the feed port.

Experimental Diets

Experiment 1. Three diets were formulated to achieve various forage:concentrate ratios using corn silage, dry-rolled corn (DRC), dried distiller grains with solubles (DDGS) and grass hay. Corn silage was considered to be 75% forage on a DM basis. Diet 1 (50:50; forage:concentrate) included 46.5% corn silage, 23.5% DRC, 15% DDGS and 15% grass hay. Diet 2 (30:70; forage:concentrate) consisted of 34% corn silage, 51% DRC, 10.5% DDGS and 4.5% grass hay on a DM basis. Diet 3 (10:90; forage:concentrate) included 11% corn silage, 74% DRC, 13.5% DDGS and 1.5% grass

hay on a DM basis. Chemical and ingredient composition of diets are provided in Table 1.

Experiment 2. A diet was formulated to achieve a 40:60 forage:concentrate ratio using 42% corn silage, 22% alfalfa haylage, 20% ground corn, 20% soybean meal and 3% vitamin and mineral supplement on a DM basis (Table 2). Corn silage was considered to be 50% forage on a DM basis.

All diet ingredients were dried in a forced-air oven for 48 h and subsequently ground. Ingredients for Exp. 1 were ground to 1 mm in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA). Ingredients were added in their respective levels into a sealable, 1-gallon bag and mixed by inversion. Diet ingredients for Exp. 2 were ground to 2 mm in the same mill and mixed in a Hobart H-600T mixer (Hobart Corporation, Troy, OH). Experimental diets and ingredient DM were measured following drying in a 100 °C oven for 24 h. Diet DM was measured on the day of inoculation for both experiments and again on d 7 of all periods in Exp. 2. Ash was determined by using a 24 h combustion in a 550 °C muffle furnace (AOAC, 2005). Neutral detergent fiber and ADF in the experimental diet and ingredients was determined with sequential detergent treatments in an ANKOM^{200} fiber analyzer (Ankom Technology, Madedon, NY). The Kjeldahl method (AOAC, 1990) was used to calculate crude protein (6.25 × N) of experimental diet and ingredients. Crude fat was measured with an ANKOM^{XT10} extractor with petroleum ether as the solvent (AOCS, 2017).

Collection of Rumen Fluid Inoculum

University of Minnesota Institutional Animal Care and Use Committee approved the use of animals for this study. Three ruminal cannulated Holstein steers served as donors for rumen fluid. The diet fed to steers was formulated to have a forage:concentrate ratio of 30:70. Diet composition can be found in Table 1.3. Steers were adapted to the diet for 21 d prior to the first rumen fluid collection. Rumen fluid was collected and transported to the laboratory in pre-warmed thermoses. After straining through four layers of cheesecloth, rumen contents from each steer were combined and homogenized under constant gassing of CO_2.

In Vitro Batch Culture Operation

Batch culture operation was slightly modified from that explained by Colombatto et al. (2003b). Approximately 0.5 g of diet DM was weighed into preweighed, acetone-washed filter bags (F57, Ankom Technology, Macedon, NY). Bags were heat-sealed and placed in 125 mL serum bottles. At the time of inoculation, 40 mL of pre-warmed anaerobic buffer medium (Goering and Van Soest, 1970) was added to serum bottles. Ten mL of rumen fluid were added to the bottle followed by 0.2 mL of appropriate enzyme solution. Bottles were capped with rubber butyl stoppers and placed randomly in a 39°C water bath. Three replications per treatment were prepared. After inoculation of all serum bottles, accumulated gas in the bottles was released and bottles were crimp-sealed with an aluminum cap. Negative controls (empty bag, rumen inoculum and buffer) were included with 3 replications. Negative control served to correct for gas production and microbial attachment to the filter bag resulting from ruminal fluid and buffer medium.

Gas production was measured at 3, 6, 9, 12, 18, 24, 30 and 36 h after inoculation by way of a 50 mL inverted burette as described by Huang et al. (2017). Bottles were

gently swirled after gas measurement and returned to the water bath. After the last gas measurement, serum bottles were drained, filter bags were removed from the bottles with tweezers. Bags were washed with cold tap water with minimal mechanical disturbance. Bags were dried at 100 °C for 24 h to determine apparent DM degradability (DMD). *In vitro* total dry matter digestibility (IVTDMD) was determined sequentially on the same filter bags using the ANKOM^{200} fiber analysis system (Ankom Technology, Macedon, NY) using procedures outlined by Van Soest et al. (1991). Sodium sulfite and α-amylase were used in NDF analysis. Rates of gas production were calculated as net volume of gas (corrected for buffer and ruminal fluid contribution) divided by each time point the gas was measured and corrected for substrate DM. Partitioning factors (PF) were calculated as the ratio of mg of DM truly digested to the mL of gas produced by digestion (Blummel and Lebzien, 2001).

Continuous Culture Operation

Eight continuous culture fermenters described by Hannah et al. (1986) were modified to include autonomous pH measurement and control system. The experiment was run in three consecutive 10 d periods with 7 d of adaptation and 3 d of sampling. Diet was hand-added to fermenters 3 times daily at 0700, 1500, and 2300 h. Feed was supplied at a rate of 75 g DM/fermenter/d. Artificial saliva (Weller and Pilgrim, 1974) was prepared to provide concentrations (g/L) of $NaHCO_3$, 5.0; Na_2HPO_4, 1.76; $KHCO_3$, 1.6; KCl, 0.6; $MgSO_4$, 0.05; and urea 0.4. Liquid and solid dilution rates of fermenters were calibrated to achieve 10%/h and 5.5%/h, respectively. Dilution rates were controlled by manipulating input of artificial saliva and output of liquid through filters. The pH of each fermenter was measured and maintained between 5.6 and 6.4 by automated addition

of 5N NaOH or 3N HCl. The pH was measured and recorded every 15 s by an electronic data acquisition software (DASYLab v5.04, Measurement Computing, Norton, MA). Anaerobic conditions were maintained by constant purging of N_2 gas at 20 mL/min. Agitation of fermenter contents was accomplished through a magnetic stir plate 350 rpm. Temperature of fermenters was maintained at 38.6 ± 0.5°C.

Samples were collected on the final 3 d of each period. Solid and liquid effluents were each collected in separate containers and remained in an ice-water bath (5 ± 5 °C) to reduce fermentative and enzymatic activity. On sampling days, effluents were combined within fermenter and homogenized using a PT10-35 homogenizer (Brinkmann Instruments, Westbury, NY). Five hundred mL of homogenized effluent was frozen at -20°C each of the 3 sampling days such that all 3 sampling days were composited by fermenter. A portion of this composite was lyophilized for analysis of DM, OM, NDF, ADF, ash and purines. Remaining effluent was subsampled for analysis of VFA, N and NH_3-N. After completion of effluent collection on 3 d of the sampling period, fermenter contents were filtered through four layers of cheesecloth. Filtered fluid was then centrifuged at 1,000 × *g* to remove feed and other larger particles. The supernatant was subsequently centrifuged at 20,000 × *g* to isolate microbial cells. Microbial cells were lyophilized for DM, OM, total N and purine analysis.

Ash and DM content of lyophilized effluent and microbial cells and experimental diets were determined by drying for 24 h in a 100 °C oven followed by a 24 h combustion in a 550 °C muffle furnace (AOAC, 2005). Ammonia-N was determined from supernatant of centrifuged (5,000 × *g*) effluent with steam distillation with MgO using a Kjeltec 2300 Analyzer Unit (Foss Tectator AB, Höganäs, Sweden). Total effluent

N was determined using Kjeldahl method (AOAC, 1990). Purine concentration of effluent and microbial pellets was determined by methods described by Zinn and Owens (1986). A NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) was used to determine RNA concentration from which purine concentration was calculated. Purine concentration of effluent and microbial cells, when compared to the N content, is used to determine the flow of bacterial N and OM in effluent samples. Concentrations of NDF and ADF in the effluent were completed using sequential detergent treatments as previously described.

Gas chromatography was used to determined effluent VFA concentrations. Before analysis, effluent was centrifuged for 15 min at 10,000 × *g*. Supernatant was hydrolyzed using a meta-phosphoric acid and crotonic acid solution and frozen overnight. After defrosting, the hydrolyzed solution was centrifuged at 10,000 × *g* for 15 min. Ethyl acetate was added to the supernatant in a 2:1 ratio, shaken vigorously and allowed to settle. The resulting ethyl acetate layer was transferred to a vial for analysis in an Agilent 7820A gas chromatograph with a 25m × 0.32mm × 0.45mm, CP-Wax 58 FFAP CB column (Agilent Technologies, Santa Clara, CA). Conditions in the chromatograph were as follows: injection volume-1.0 µL; injector temperature-200 °C; flame ionization temperature-220 °C; carrier gas (N_2) flow-26.2 kPa. Oven temperature was 110 °C and ramped to 220 °C. Standard solutions with known concentrations of VFA were prepared and analyzed under identical conditions. Standard curves were used to determine VFA concentrations of effluent samples.

Statistical Analysis

All data were analyzed in SAS 9.4 statistical software (SAS Institute Inc., Cary, NC). Experiment 1 was analyzed as a completely randomized design. A linear additive model for each dependent variable was:

$$Y_{ijk} = \mu + D_i + E_j + (DE)_{ij} + A_k + e_{ijk}$$

Where μ is the overall mean, D_i is the effect of diet, E_j is the effect of enzyme treatment, $(DE)_{ij}$ is the interaction between diet and enzyme treatment, A_k is the random effect of assay and e_{ijk} is the error term. The effect of dose was not significant ($p > 0.05$); therefore, it was removed to facilitate model interpretation. Data for DMD, IVTDMD, gas production and PF were analyzed using PROC MIXED procedure and differences between treatment means calculated with Tukey-Kramer-adjusted LSMEANS option. Results are thus reported as least squared means from 15 observations per treatment (three replicates and five doses). Rate of gas production was analyzed using PROC MIXED procedure of with measurements taken at 3, 6, 9, 12, 18, 24, 30 and 36 h after inoculation. Repeated measures analysis was performed using spatial power covariance structure. The model included fixed effects of diet, enzyme, time and all two and three-way interactions. Tukey-Kramer adjustment was used on differences of least square means, which were sliced by the interaction term of enzyme, diet and time.

Data from Exp. 2 were analyzed as a randomized complete design. The linear additive model was:

$$Y_{ij} = \mu + E_i + P_j + e_{ij}$$

Where μ is the overall mean, E_i is the effect of enzyme treatment, P_j is the random effect experimental period and e_{ij} is the error term. Analysis was performed using PROC

MIXED procedure of SAS for all dependent variables. Differences between treatment means were tested using LSMEANS option in SAS. Tukey-Kramer adjustment was used to determine differences between treatment means. All results are reported as least square means with six observations per treatment.

Fermenter pH was analyzed in PROC MIXED of SAS. pH was measured every 15 s during the 3 d of sampling for period. The recorded pH was averaged by hour over the 3 d sampling period. Repeated measures analysis was performed with a first-order autoregressive covariance structure. The model treated enzyme treatment, time and the interaction of treatment and time as fixed effects and period and fermenter nested within period as random effects. Least square means were sliced by the interaction of treatment and time. Tukey-Kramer-adjusted least square means were used to determine differences between treatments on an hourly basis.

RESULTS AND DISCUSSION

Experiment 1.

Results for DMD are presented in Figure 1. Interaction between enzyme and diet and the main effects of diet and enzyme were significant ($P < 0.05$). Within each enzyme, DMD increased ($P < 0.05$) from Diet 1 to Diet 2 and from Diet 2 to Diet 3. Across all enzyme treatments, Diet 2 had greater ($P < 0.05$) apparent DMD than all enzyme treatments in Diet 1. Similarly, most enzyme treatments in Diet 3 had a greater ($P < 0.05$) DMD digestibility than enzyme treatments in Diet 2 except for no difference ($P > 0.05$) between CON in Diet 3 and PRO and AAM in Diet 2. Differences between diets were expected due to an increase in levels of concentrates in Diets 2 and 3. This observation is consistent with Weiss and Shockey (1991) who found that digestibility of DM increases

with increasing levels of concentrate. Ruminal digestibility of starch from dry-rolled corn was estimated to be 76.2% of intake (Huntington, 1997). Forage NDF, which was present in the grass and corn silage at 75.4 and 33.3 (%DM), respectively, is less than 50% digestible in the rumen (Van Soest, 1994).

Within diet, there were no differences ($P > 0.05$) in variables measured due to enzyme product, with a few exceptions. In Diet 1, PRO supplementation increased ($P < 0.01$) apparent DMD compared with BGL. In a similar experiment, Eun et al. (2006) found that a protease enzyme increased degradability of rice straw. Colombatto et al. (2003a,b) discovered that protease enzymes increased DM and NDF degradability of alfalfa hay and TMR *in vitro.* In Diet 3, FAE increased ($P < 0.01$) DMD compared with CON, and it tended ($P = 0.064$) to increase DMD compared with PRO supplementation. Ferulic acid esterase was studied as a pretreatment to forages (Nsereko et al., 2000; Krueger and Adesogan, 2008). An enzyme mixture containing ferulic acid esterase increased DM digestibility in cows fed low and high concentrate diets (Arriola et al., 2011). Ferulic acid esterase hydrolyze ester linkages between hydroxycinnamic acids and sugars in plant cell walls and opens other polysaccharides in the cell wall for further enzymatic hydrolysis (Yu et al., 2005). Effects of ferulic acid esterase were enhanced when supplemented with a mixture of enzymes (Bartolome et al., 1995). Results for DMD may be difficult to evaluate due to microbial and debris attachment to feed substrate and filter bags. Removal of these contaminants from samples can be accomplished using neutral detergent solution after batch culture incubation to obtain "true digestibility" (Goering and Van Soest, 1970).

Data for IVTDMD are presented in Figure 2. As with DMD, IVTDMD was affected by diet, enzyme and the interaction of diet and enzyme. Within Diet 1, BGL treatment increased ($P < 0.05$) IVTDMD compared to all other enzyme treatments and CON. Similarly, in Diet 2, BGL increased ($P < 0.05$) IVTDMD compared to other treatments. In Diet 3, BGL treatment increased ($P < 0.05$) IVTDMD compared to CON and AAM. Overall, BGL demonstrated an ability to increase IVTDMD. For clarification, cereal β-glucans are consecutively linked β-(1-4) glucose polymers (oligomeric cellulose segments) that are separated by single β-(1-3) linkages (Lazaridou and Biliaderis, 2007). These polymers are common in cereal grains such as oats, barley and wheat (Savelkoul et al., 2013). Because of the broad definition of β-glucans, cellulose is also considered a β-glucan (Bacic et al., 1988) As no oat, barley or wheat were used in this experiment, it is possible BGL had substantial endo-β-1,4-glucanase and exo-β-1,4-glucanase activity to hydrolyze cellulose versus β-1,3-glucanase which would digest cereal β-glucans. Determination of specific enzymatic activities of enzyme treatments would help discern the actual activities present in enzyme products. Many enzyme preparations have activities different from how they are marketed (Colombatto et al., 2003b). Fibrolytic enzymes with varying endoglucanase, xylanase, and exoglucanase activities improved *in vitro* NDF degradability. Endoglucanase and exoglucanase accounted for more than 70% of total variation in NDF degradability for alfalfa hay and corn silage (Eun and Beauchemin, 2007b). Due to the nature of the assay, IVTDMD may bias enzyme treatments which target cell wall components. Neutral detergent treatment following batch incubation will also degrade undigested starch, pectin, protein and other non-

structural components. Therefore, differences in digestion of non-cell wall components cannot be detected with IVTDMD.

Main effects of diet and enzyme treatment were significant ($P < 0.05$) for total gas production and are presented in Figure 3 and 4, respectively. The interaction of enzyme and diet was not significant ($P > 0.05$) for this variable. Total gas production increased ($P < 0.05$) from Diet 1 to Diet 2 and from Diet 2 to Diet 3. These results contradict Eun et al. (2004) who found no differences in total gas production with varying forage:concentrate ratios in continuous culture. In batch culture, Kumar et al. (2013) also found no differences in total gas production between three diets with various forage:concentrate ratios. However, Menke and Steingass (1988) related gas production *in vitro* to feedstuff digestibility. In this experiment, when concentrate in the diet and DMD increased so did total gas production. However, this logic is contradicted by the effect of enzyme treatment on gas production. If the correlation between gas production and feed degradability remained true, BGL would be expected to have greater gas production due to its significant increase in IVTDMD. Conversely, CON and XYL had higher ($P < 0.05$) total gas production than MEB, FAE and AAM. BGL and PRO gas production was also larger ($P < 0.05$) than MEB. Phakachoed et al. (2013) observed a similar contradiction. Although fibrolytic enzymes increased NDF and ADF disappearance, no differences were observed in total gas production (Phakachoed et al., 2013) These results disagree with those observed by Eun and Beauchemin (2007a) who demonstrated an increase in OM digestibility that corresponded with an increase in total gas production. Differences between the current experiment and previous work may be attributed to encapsulation of

feed substrate in a filter bag which can decrease accessible surface area for microbial attachment and hydrolysis and may change gas production kinetics and feed degradation.

Rates of gas production for Diet 1, Diet 2 and Diet 3 are displayed in Figure 5, 6 and 7, respectively. Within each diet there were no differences ($P > 0.05$) in rate of gas production from 24 to 36 h between any enzyme treatments. In addition, Colombatto et al. (2007) did not find differences in gas production past 19 h of incubation. In Diets 1 and 2, BGL and AAM were calculated to have a negative rate of gas production as they accumulated less gas than the negative control (buffer and rumen inoculum) in 3 h of fermentation.

In general, initial rate of gas production (3 h) was fastest for CON and slowest for BGL and AAM. This is intriguing due to the vastly higher IVTDMD for BGL over CON. Seemingly, enzyme treatments influence the kinetics of gas production. A clear pattern after 3 h is difficult to discern because of significant interactions of diet, enzyme and time. Eun and Beauchemin (2007a) also examined the effect of enzymes on the rate of gas production *in vitro.* Their incubation was only 18 h and the feed substrates were not contained in a filter bag as in the current experiment. They determined enzyme products increased rates of gas production from 2 h to 12 h. Colombatto et al. (2003c) used pure substrates of cellulose, oat spelt xylan and the two in combination to determine fermentation *in vitro.* Concordant with previous studies, they found enzyme treatment decreased the lag phase and increased the fractional rate of gas production at 6 h. Both experiments support the hypothesis that a mode of action of EE is to increase the rate of fermentation in the early stages of fermentation allowing rumen microbes earlier access to fermentable substrates (Colombatto et al., 2003c). It is possible enzyme treatment in

this experiment initially competed with endogenous enzymes for binding cites on plant cell walls explaining CON's fast initial rates of gas production (Morgavi et al., 2001).

Partitioning factor, the ratio of mg of DM digested to mL of gas produced, is a measure of carbon partitioning and may estimate efficiency of microbial protein synthesis (Blümmel et al., 1997). Data for PF is shown in Figure 8. Interaction of enzyme and diet was significant ($P < 0.05$). In Diet 1, BGL was larger ($P < 0.05$) than CON and XYL. Within Diet 2, BGL tended ($P = 0.074$) to be larger than AAM. FAE was larger ($P < 0.05$) than CON in Diet 3. Partitioning factor reflects partitioning of carbons from degraded substrates to gas production and can also be used to estimate short-chain fatty acid production or microbial protein synthesis (Blümmel et al., 1997). Although PF is not known to be used as a parameter to evaluate the effects of EE *in vitro*, it may be a useful tool to select enzymes which shift fermentation to more favorable end-products. Values for PF in the current experiment ranged from 3.09 to 4.54 and align with those reported by Blummel et al. (1997) that ranged from 2.74 to 4.65 for a variety of feed substrates. Several enzymes may shift the fermentation of substrate from gas production to either VFA or microbial protein compared with no enzyme treatment but depends on the diet and type of enzyme.

The objective of Exp. 1 was to screen enzyme products for further evaluation in continuous culture. Therefore based on the results, BGL and PRO were selected for further analysis. BGL had higher IVTDMD and as well as a higher PF in Diet 1 than CON. Considering no other treatment showed consistent improvements in digestibility or PF, PRO was selected because it had the second largest numerical effect on IVTDMD.

Experiment 2.

Fiber, OM, and DM digestibility results are presented in Table 4. Enzyme supplementation did not influence ($P > 0.05$) true DM or OM digestibility. CON tended to have a greater ($P = 0.067$) apparent DM than CEL. CON also tended to have a greater ($P = 0.092$) apparent OM digestibility compared with CEL. This corresponded with numerical increases ($P > 0.05$) in NDF and ADF digestibility for CON over CEL. Although previous research has been variable, these results contradict results from Exp. 1 as well as previous CC experiments with enzyme supplementation. Vera et al. (2012) used a proteolytic enzyme in diets with or without DDGS. In the diet with DDGS, enzyme supplementation increased DM, OM and NDF. The interaction between diet and the enzyme's effect on digestibility may correspond to lower lignin content of DDGS compared to the forage counterparts used in Exp. 2. Lower lignin content can lead to easier access of the target substrates for proteolytic enzymes. Colombatto et al. (2003a) conducted an experiment to determine the effect of pH on the efficacy of enzyme addition in CC. Enzyme supplementation increased NDF degradation especially in the high pH range. Similar to these results, Yang et al. (2002) found NDF degradation increased with enzyme supplementation with the increase being more substantial when pH was maintained above 6.0. Interestingly, the enzyme used in that study demonstrated optimal fibrolytic activity when tested under acidic conditions.

In the current experiment, pH was not different ($P > 0.05$) between any treatments. Although the pH was allowed to vary between 5.6 and 6.4, pH of all four treatments across the 3 d sampling period averaged 5.74 (data not shown). This corresponded closely to the low pH treatments used by Yang et al. (2002) and Colombatto et al. (2003a). Cellulolytic activity of microorganisms can be compromised

when pH is lower 6.2 (Russell and Wilson, 1996), yet the pH optima for many commercial fibrolytic enzyme activities' range from 4.5 to 5.5. Based on findings from the current experiment and that of Yang et al. (2002) and Colombatto et al. (2003a), lower pH environments may decrease the efficacy of enzyme supplementation compared with their higher pH counterparts. These findings contradict previous theories which expected effects of fibrolytic enzymes to be greater in environments where endogenous enzyme capacity is limited. These environments include when pH is low or with high rates of passage (Romero et al., 2016).

Concentration of VFA and ratio of acetate to propionate ratio (A:P) in fermenter effluent is presented in Table 5. Total VFA concentration was not affected ($P > 0.05$) by enzyme supplementation. Molar proportions of individual VFA, acetate to propionate ratio and branched-chain VFA concentration were also not affected ($P > 0.05$) by enzyme supplementation. Results from similar experiments have been variable with total VFA concentration increasing in several experiments. Proteolytic enzymes in lactating dairy cow diets (Eun and Beauchemin 2005), fibrolytic enzymes in high-forage *in vitro* diets (Giraldo et al., 2008) and polysaccharide-degrading enzymes in grain-based heifer diets (Hristov et al., 2000) all resulted in increases in total VFA concentrations compared with controls. However, enzyme supplementation did not affect total VFA concentration of CC fermentation with an enzyme mixture (Colombatto et al., 2003a) or in dairy cows with a polysaccharide-degrading enzyme (Hristov et al., 2008).

There was no difference ($P > 0.05$) in any individual VFA concentration between treatment groups (Table 5). This observation contrasts with Tricarico and Dawson (2005)

who found endoglucanase and endoglucanase-xylanase enzymes significantly reduced the A:P ratio in 12 h batch cultures.

Nitrogen metabolism was not affected ($P > 0.05$) by enzyme treatment (Table 6). Ammonia-N concentrations ranged from 4.82 to 6.61 but were not affected ($P > 0.05$) by enzyme treatment. Flow of NH_3-N, non-NH_3-N, microbial-N and dietary-N were also unaffected ($P > 0.05$) by enzyme treatment. There was also no effect ($P > 0.05$) of enzyme treatment on efficiency of microbial protein synthesis expressed as g of microbial N/kg of OM truly digested. Colombatto et al. (2003a) found that total N flow decreased with enzyme addition while NH_3-N concentrations tended to increase with enzymes. Neither bacterial nor dietary N flows were affected by treatment. Ammonia-N concentrations were extremely low compared to similar CC experiments (Erfle et al., 1982; Calsamiglia et al., 2002). The authors did not believe ammonia was limiting due to adequate protein degradation and branched-chain VFA production (Colombatto et al., 2003a). Yang et al. (2002) found that enzymes had no effect on microbial protein synthesis or CP degradation. In an *in vivo* experiment with a similar enzyme product to Yang et al. (2002), Beauchemin et al. (1999b) found enzyme supplementation increased proportion of microbial N in non-NH_3-N which coincided with an increase in fiber digestion in dairy cows. Yang et al. (1999) also detected an increase in microbial protein synthesis in dairy cows fed an enzyme mixture.

Experiment differences

The objective of Exp. 1 was to screen enzyme products which had the greatest effects on feed degradability. Therefore, enzymes chosen to be evaluated in continuous culture were hypothesized to influence fermentation. Experiment 1 demonstrated that

enzymes may affect rates of gas production, substrate degradability, and partitioning of carbon. In contrast, Exp. 2 deduced that there were few impacts of enzyme supplementation in continuous culture fermentation. Differences between batch cultures and continuous culture fermentation may explain some of the observed variation in efficacy of enzyme supplementation. Enzymes in Exp. 1 were dissolved in distilled water before being added to the serum bottles. Enzymes in Exp. 2 were top-dressed in granular form onto the diet immediately before feeding. Exogenous enzymes typically increase only the rate but not extent of feed digestion. Therefore, any positive responses seen with enzyme supplementation are not a result of exogenous enzyme hydrolyzing substrates that would not be digested normally (Meale et al., 2014). A synergistic relationship between enzyme addition and endogenous microbiota may contribute to observed benefits of enzyme addition. This relationship is the consequence of enhanced bacterial attachment to feed (Morgavi et al., 2000). Applying enzyme as a diluted solution may allow for rapid adherence of enzyme to feed substrate and swift attachment of microbes to feed. In previous CC experiments, EE were applied as a solution and an increase substrate degradability was found (Yang et al., 2002; Colombatto et al., 2003a; Vera et al., 2012). Exogenous enzymes were not bound to substrate and it may have allowed exposed exogenous enzyme to rapid proteolysis by rumen microbes (Beauchemin et al., 2003). Increased doses of exogenous enzyme may also compete for binding sites on feed with endogenous enzyme and microbes and lead to a lack of or negative effects (Morgavi et al., 2000). It is possible that fast dilution rates in Exp. 2 rapidly removed EE from the vessel, especially if EE were not bound to feed particles. If this were the case, an increase

in hydrolytic activity of effluent could lead to post-ruminal effects in an *in vivo* model (Beauchemin et al., 1999b).

CONCLUSION

Supplementation of various EE yielded variable results during batch and continuous culture fermentation. Inclusion of EE did not consistently increase digestibility or gas production in batch culture. BGL demonstrated larger IVTDMD with no increase detected in total or rate gas production compared with CON. In continuous culture fermenters, BGL, had no effects on digestibility or nitrogen metabolism or VFA production compared with CON. The highly buffered end-point fermentation in a batch culture versus the continuous flow of buffer and effluent in continuous culture fermenters may contribute to differences found in the two experiments. In order to properly apply enzymes to ruminant diets, the mechanism of action of exogenous enzyme must be determined. Use of *in vitro* assays offer a controlled and mechanistic approach to determine the efficacy of enzyme feed additives.

Table 1. Ingredient and chemical composition of Exp. 1 diets.

Item	Diet 1	Diet 2	Diet 3
Feed composition[1]			
Corn silage	46.5	34.0	11.0
Dry-rolled corn	23.5	51.0	74.0
DDGS	15.0	10.5	13.5
Grass hay	15.0	4.5	1.5
Chemical composition[1]	9.0	8.2	1.5
Crude protein	10.9	11.0	11.4
NDF	33.3	22.6	14.9
ADF	16.9	10.0	5.0
Crude fat	3.5	3.8	4.2
Ash	4.7	3.2	1.9

[1]Composition as % DM.

Table 2. Ingredient and chemical composition of Exp. 2 diet.

Item	Composition[1]
Diet composition	
Corn silage	42.0
Alfalfa haylage	22.0
Ground corn grain	20.0
Soybean meal	20.0
Vitamin and mineral supplement	3.0
Chemical composition	
Crude protein	15.2
NDF	24.0
ADF	13.5
Crude fat	3.3
Ash	7.1

[1]Composition as % of DM.

Table 3. Diet composition of donor steers.

Ingredient	Composition[1]
Corn silage	35.3
Dry-rolled corn	32.8
DDGS	14.3
Grass hay	12.7
Liquid Supplement[2]	4.9

[1]Composition as % of DM.

[2]Liquid supplement composition.

Table 4. Effects of exogenous enzyme supplementation on DM, OM and fiber digestion in continuous culture

	Treatment[1]				SEM[2]	P-value[3]
Digestion (%)	**CON**	**CEL**	**PRO**	**BGL**		
DM, apparent	51.8	42.7	44.8	45.0	2.4	0.08
DM, true[4]	71.3	62.2	64.0	63.3	3.0	0.18
OM, apparent	40.6	33.6	34.4	35.8	2.0	0.09
OM, true[4]	58.2	51.3	52.0	52.3	2.6	0.26
NDF	51.9	37.5	43.9	47.1	4.1	0.12
ADF	52.2	37.9	40.9	47.7	4.3	0.11

[1]CON: 0 mg enzyme/kg diet DM; CEL: 1000 mg Cellulase enzyme/kg diet DM; PRO: 1000 mg Protease enzyme/kg diet DM; BGL: 1000 mg Beta-glucanase/kg diet DM.

[2]Standard error of the mean, n = 6 replicates per treatment.

[3]Probability corresponding to the null hypothesis.

[4]Corrected for bacterial contribution.

Table 5. Effect of exogenous enzyme supplementation on VFA concentration in continuous culture.

	Treatment[1]				SEM[2]	P-value[3]
Volatile fatty acids	**CON**	**CEL**	**PRO**	**BGL**		
Total VFA, m*M*	116.7	118.0	116.5	120.0	2.82	0.80
Individual VFA, mol/100 mol						
Acetate	60.6	62.8	61.9	61.9	1.06	0.56
Propionate	20.1	19.2	19.3	19.3	0.77	0.81
Butyrate	12.7	11.8	13.0	12.4	0.44	0.25
Valerate	2.59	2.44	2.31	2.49	0.13	0.52
Isobutyrate	0.59	0.59	0.59	0.57	0.03	0.98
Isovalerate	1.98	1.92	1.74	1.91	0.09	0.32
A:P Ratio	3.07	3.34	3.27	3.25	0.17	0.71

[1]CON: 0 mg enzyme/kg diet DM; CEL: 1000 mg Cellulase enzyme/kg diet DM; PRO: 1000 mg Protease enzyme/kg diet DM; BGL: 1000 mg Beta-glucanase/kg diet DM.

[2]Standard error of the mean, n = 6 replicates per treatment.

[3]Probability corresponding to the null hypothesis.

Table 6. Effect of exogenous enzyme supplementation on nitrogen metabolism in continuous culture.

Item	Treatment[1]				SEM[2]	P-value[3]
	CON	CEL	PRO	BGL		
NH_3-N, mg/dl	5.52	6.04	6.61	4.82	0.86	0.52
N flow, g/d						
NH_3-N	0.14	0.16	0.17	0.12	0.02	0.54
Non NH_3-N	2.10	2.06	2.00	2.08	0.04	0.44
Microbial-N	1.10	1.11	1.14	1.06	0.11	0.95
Dietary-N	1.00	0.95	0.86	1.02	0.12	0.82
CP degradation, %	58.8	60.8	64.6	58.1	5.19	0.81
EMPS[4]	27.0	30.8	31.6	29.1	2.27	0.51

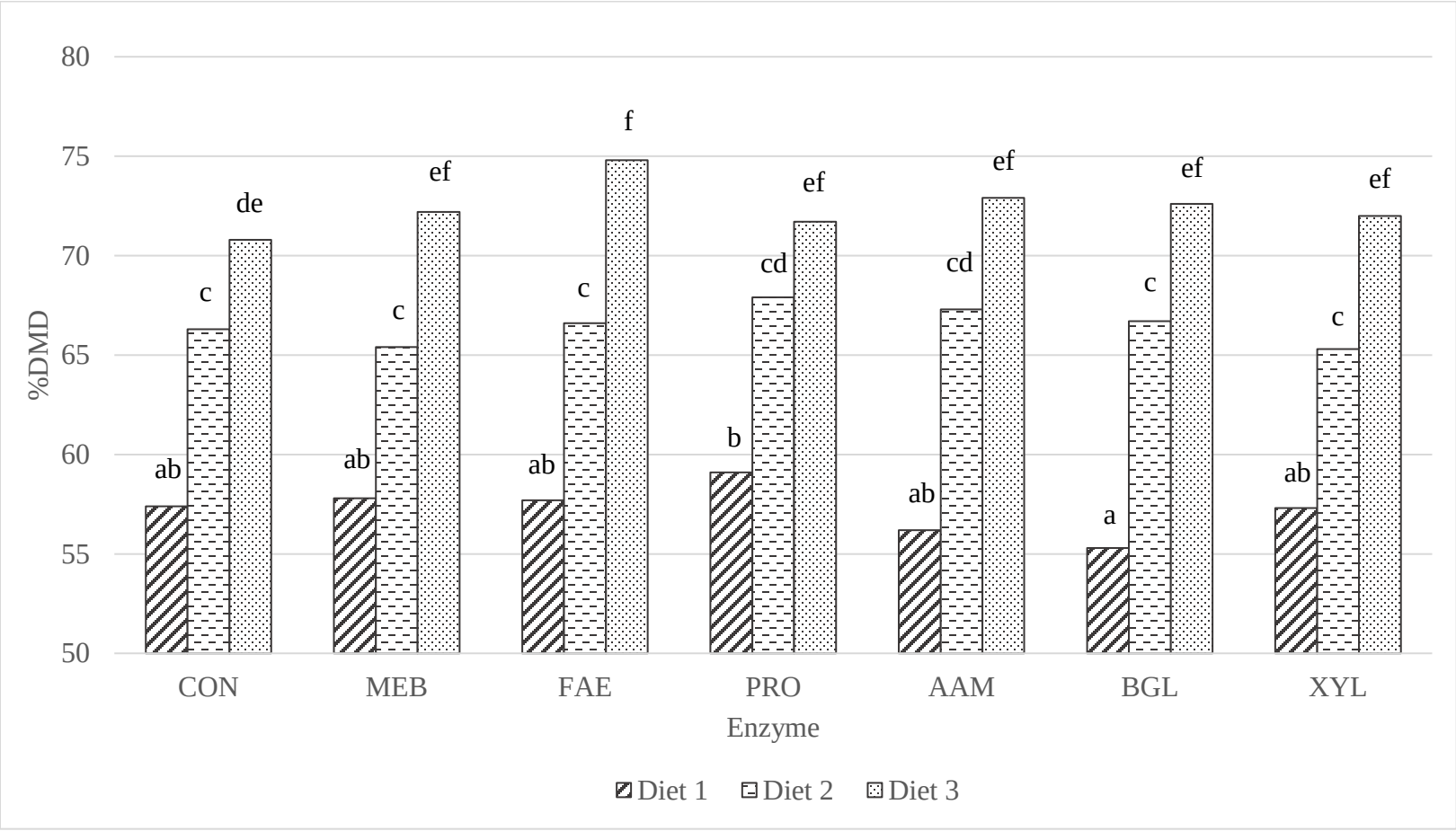
[1]CON: 0 mg enzyme/kg diet DM; CEL: 1000 mg cellulase enzyme/kg diet DM; PRO: 1000 mg protease enzyme/kg diet DM; BGL: 1000 mg β-glucanase/kg diet DM.

[2]Standard error of the mean, n = 6 replicates per treatment.

[3]Probability corresponding to the null hypothesis.

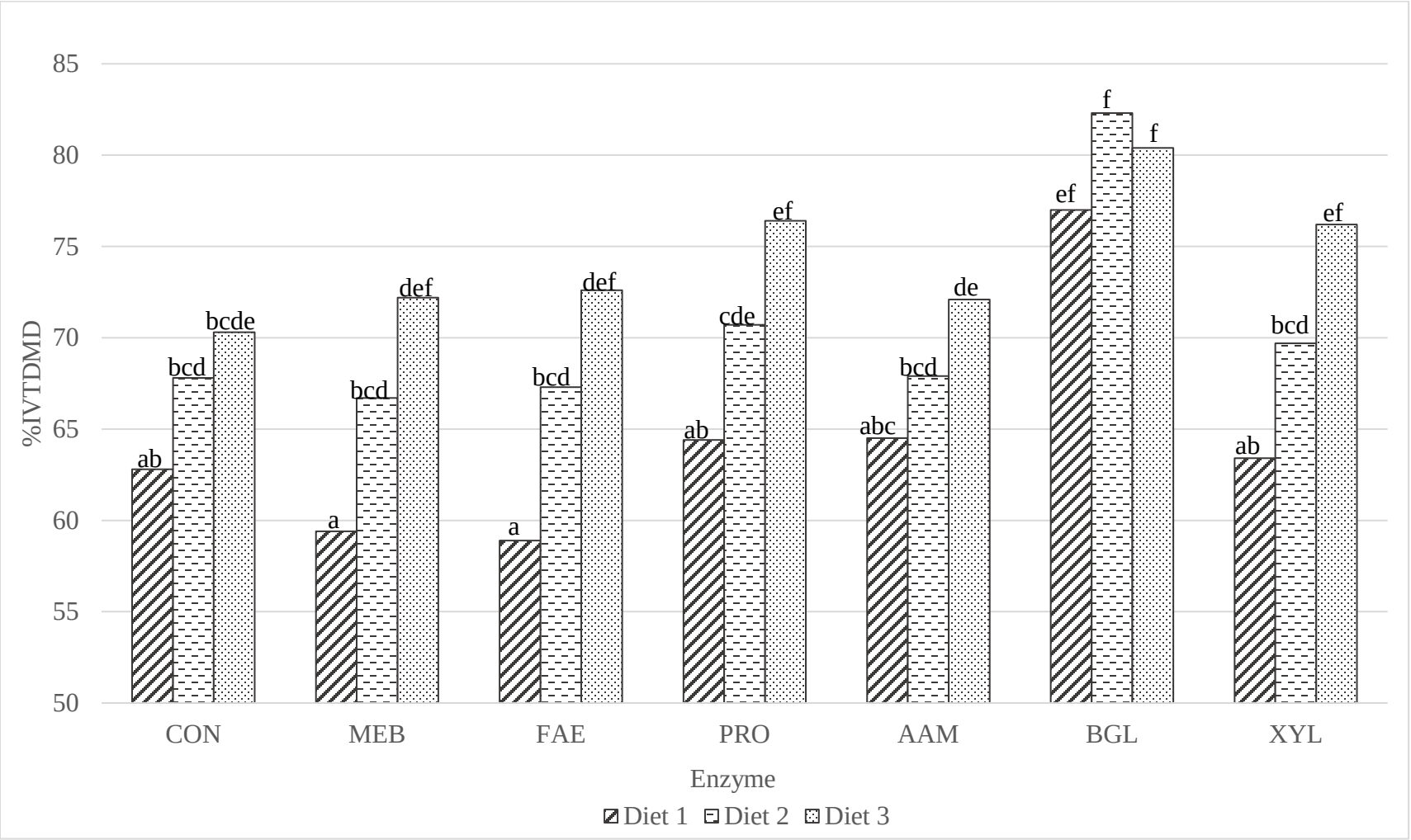
[4]EMPS: efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested).

Figure 1. Effect of enzyme supplementation on diets with varying forage:concentrate on DMD in batch culture.



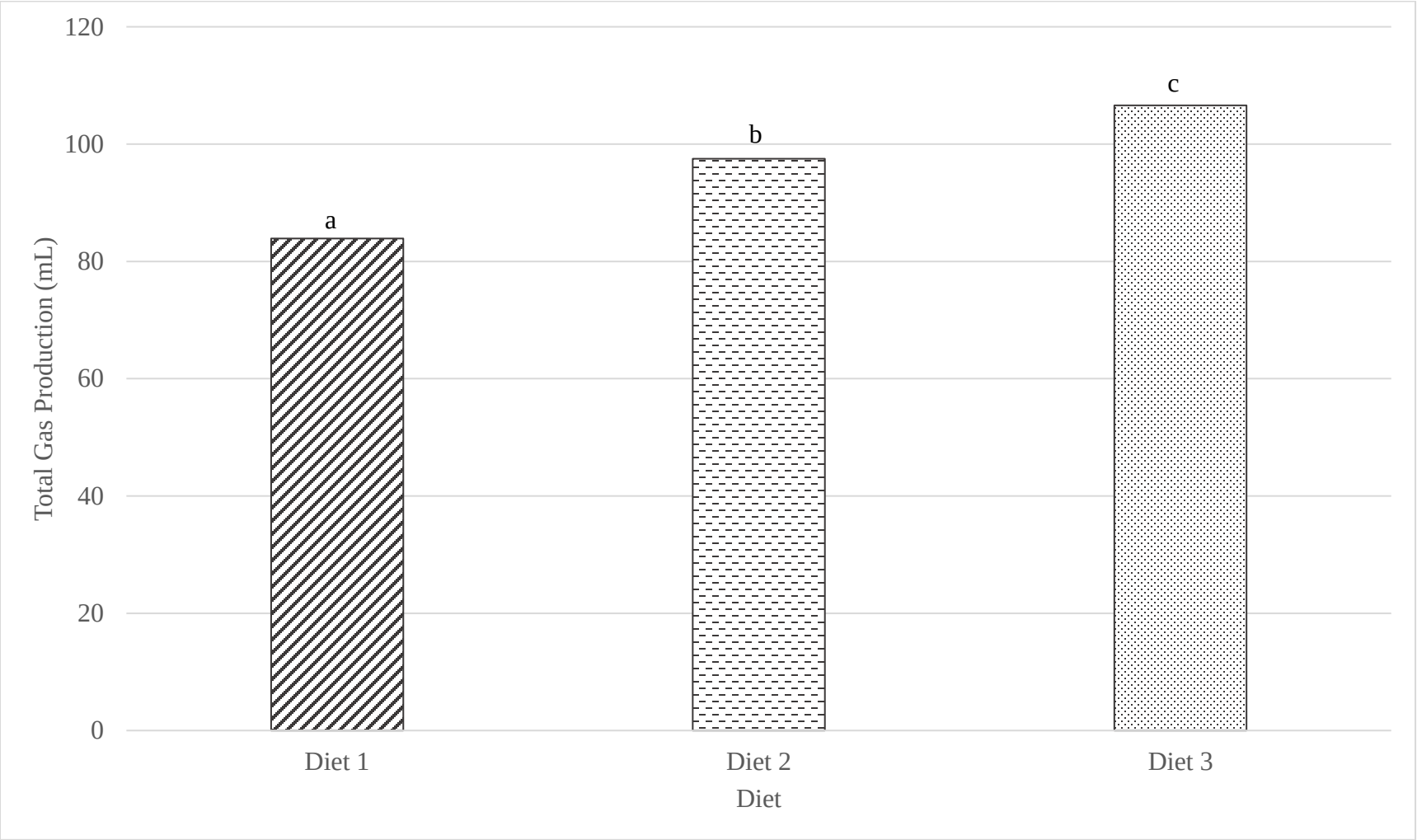
[a, b, c, d, e, f] Bars without a common superscript differ, $P < 0.05$.

Figure 2. Effect of enzyme supplementation on diets with varying forage:concentrate on IVTDMD in batch culture.



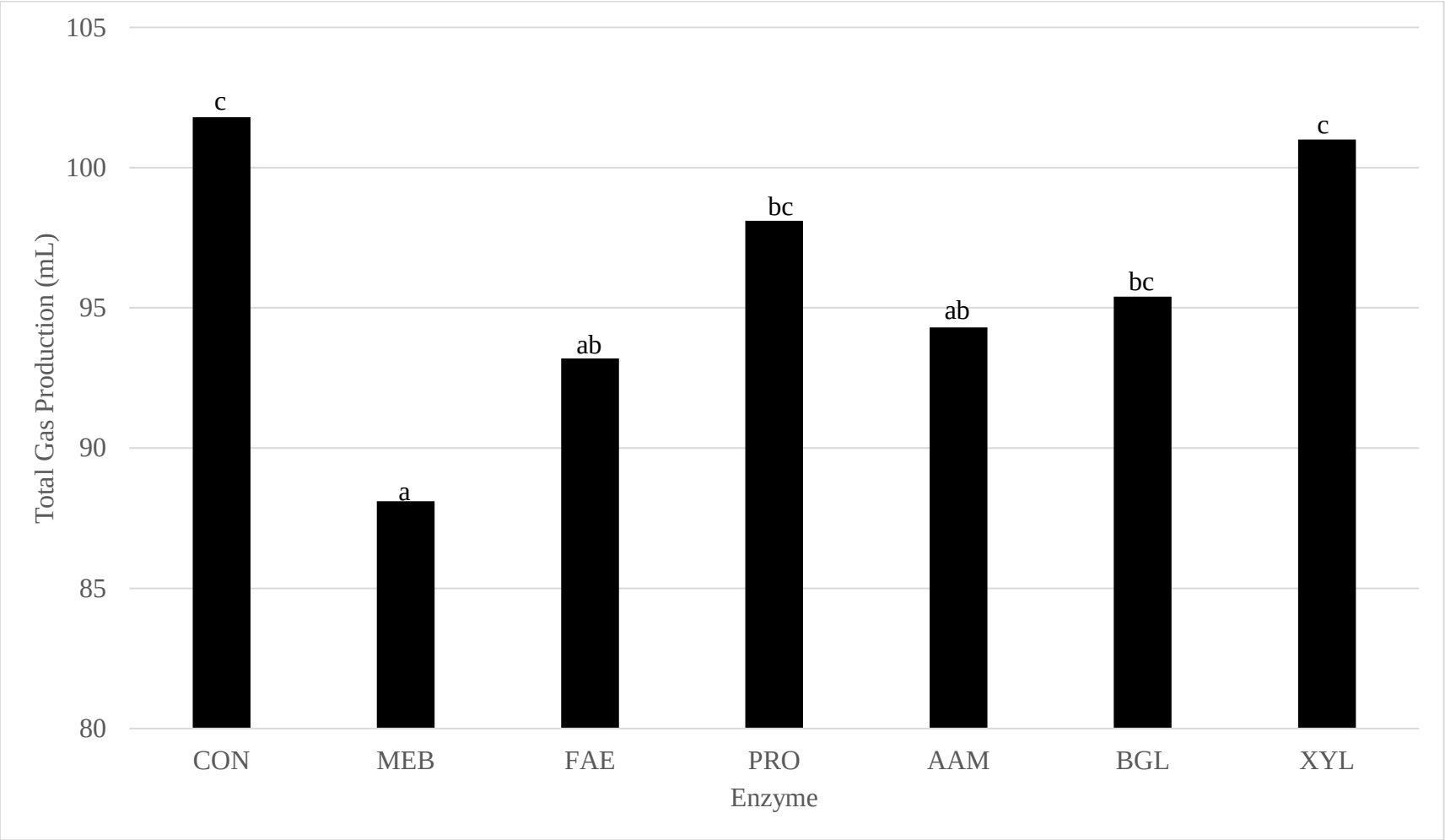
[a, b, c, d, e, f] Bars without a common superscript differ, $P < 0.05$.

Figure 3. Effect of diet on total gas production in batch culture.



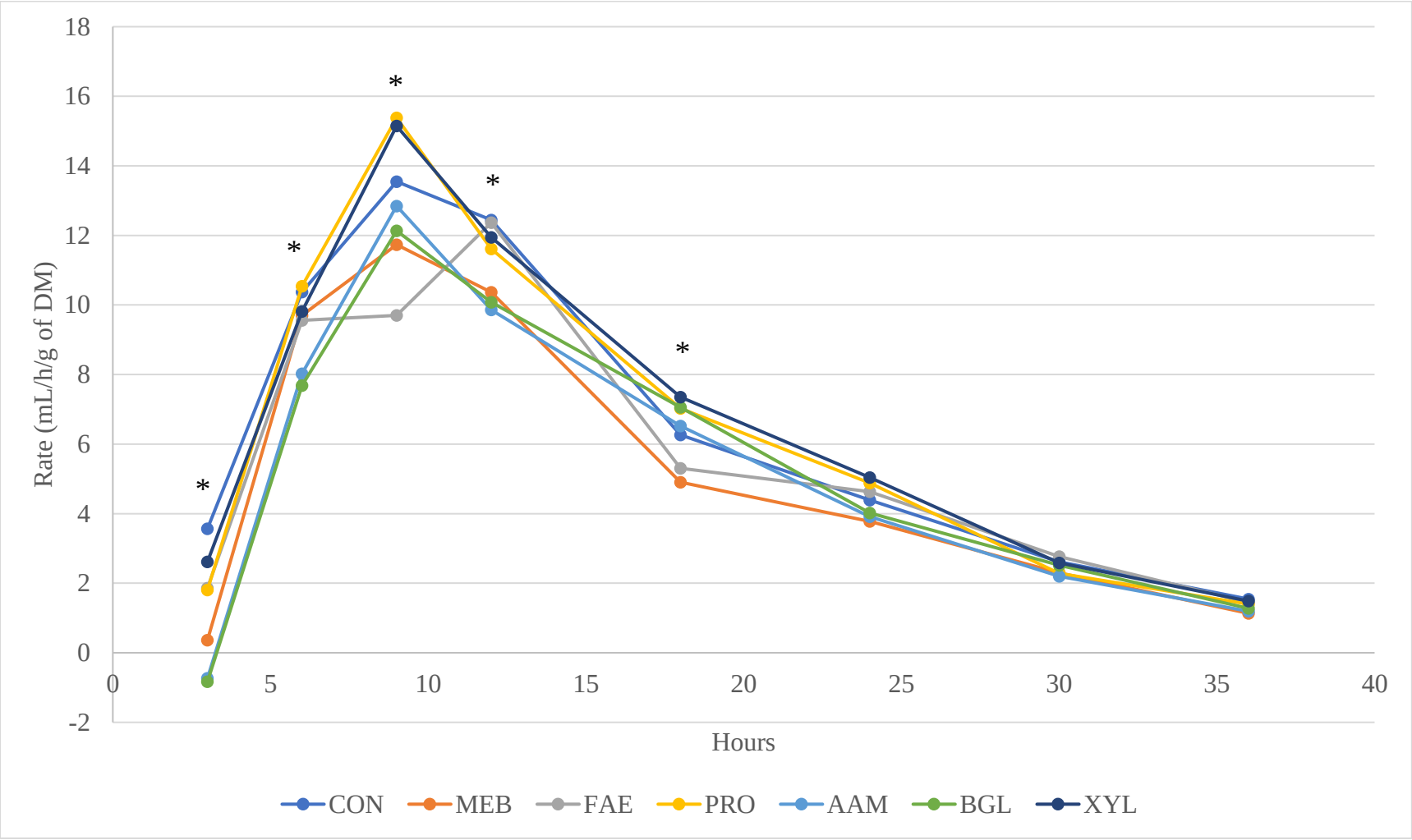
[a,b,c] Bars without a common superscript differ, $P < 0.05$.

Figure 4. Effect of exogenous enzyme on total gas production in batch culture.



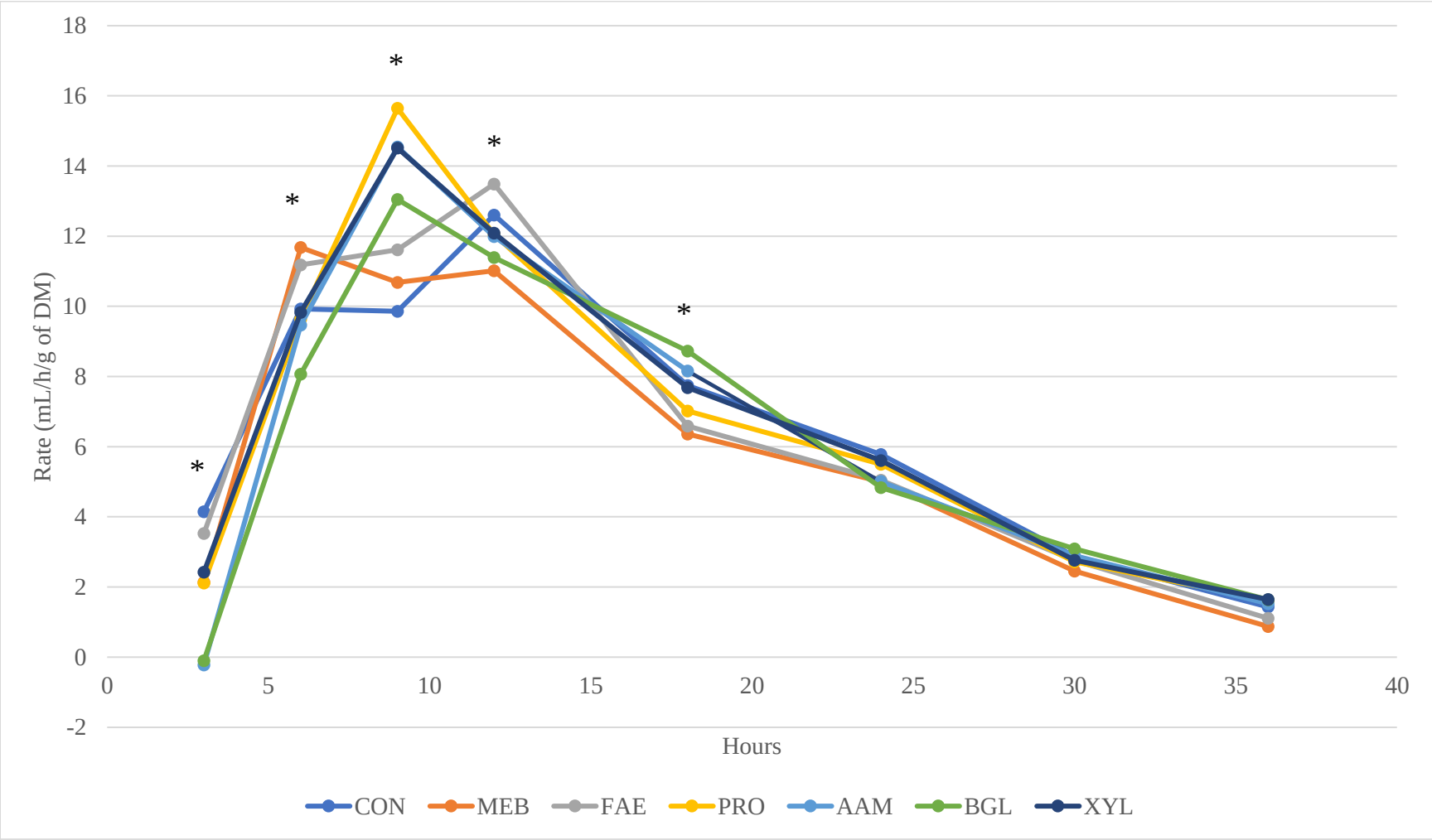
[a,b,c] Bars without a common superscript differ, $P < 0.05$.

Figure 5. Effect of exogenous enzyme supplementation on rate of gas production in Diet 1.



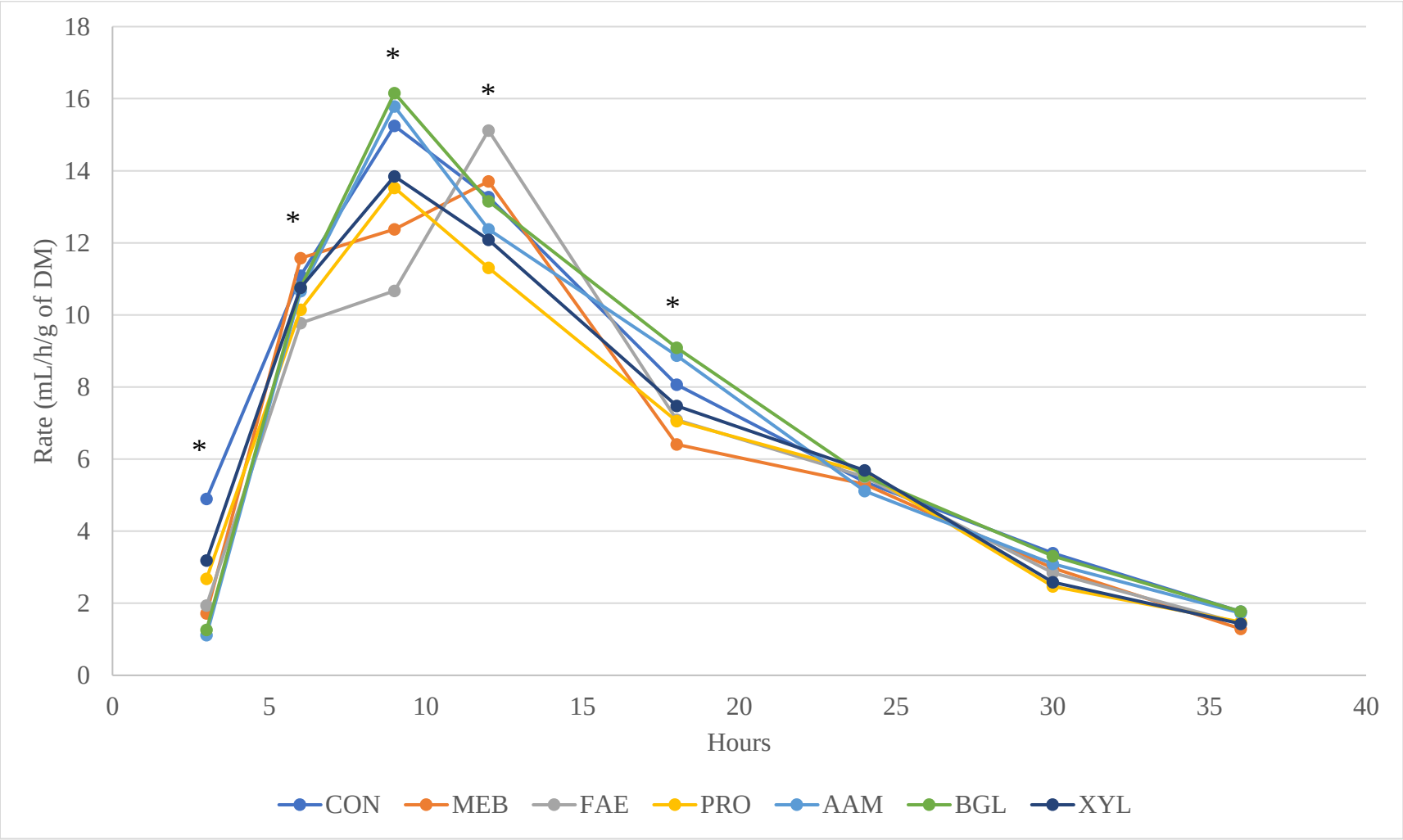
***** Signifies difference between least square means of enzyme and diet interaction, ($P < 0.05$).

Figure 6. Effect of exogenous enzyme supplementation on rate of gas production in Diet 2.



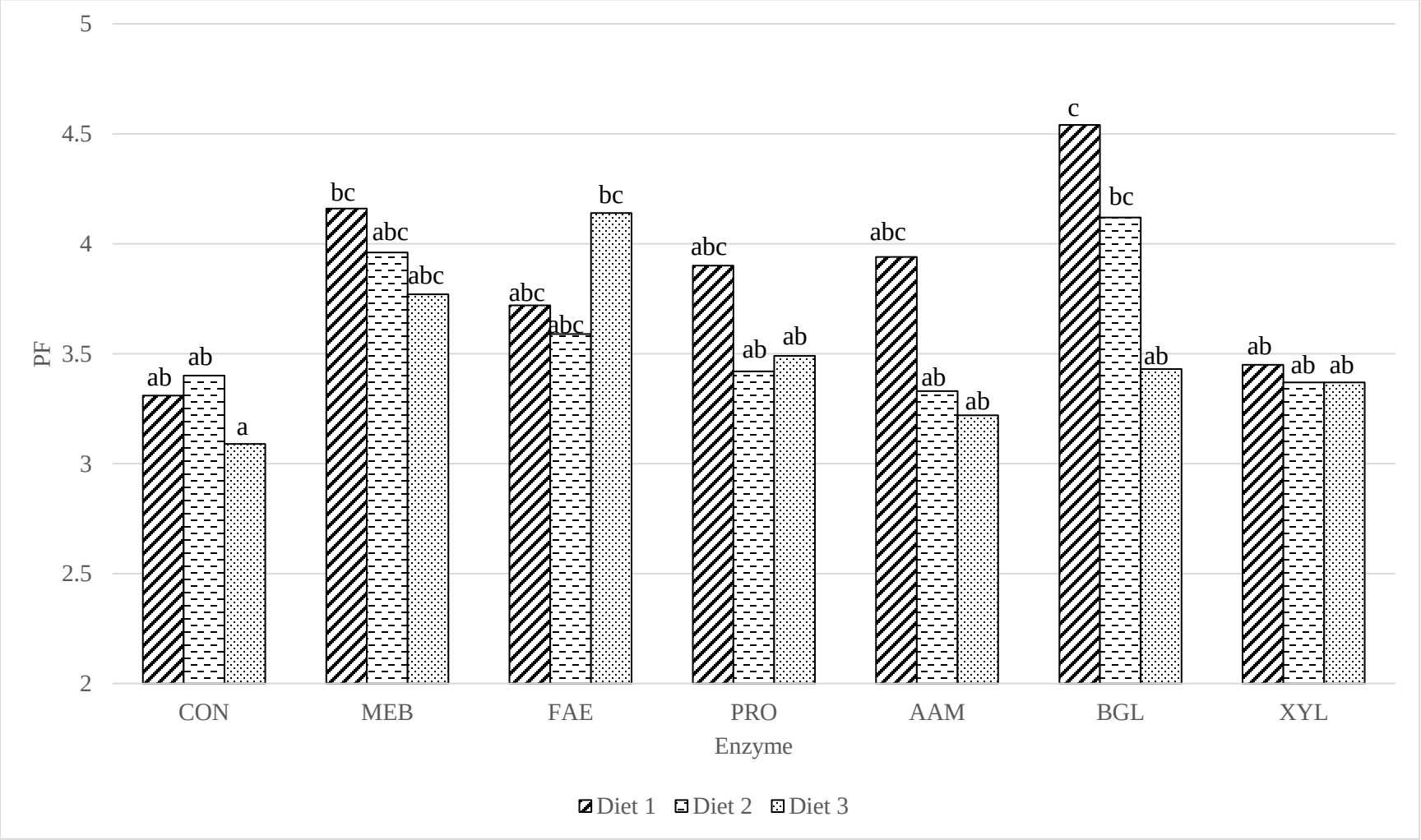
***** Signifies difference between least square means of enzyme and diet interaction, ($P < 0.05$).

Figure 7. Effect of exogenous enzyme supplementation on rate of gas production in Diet 3.



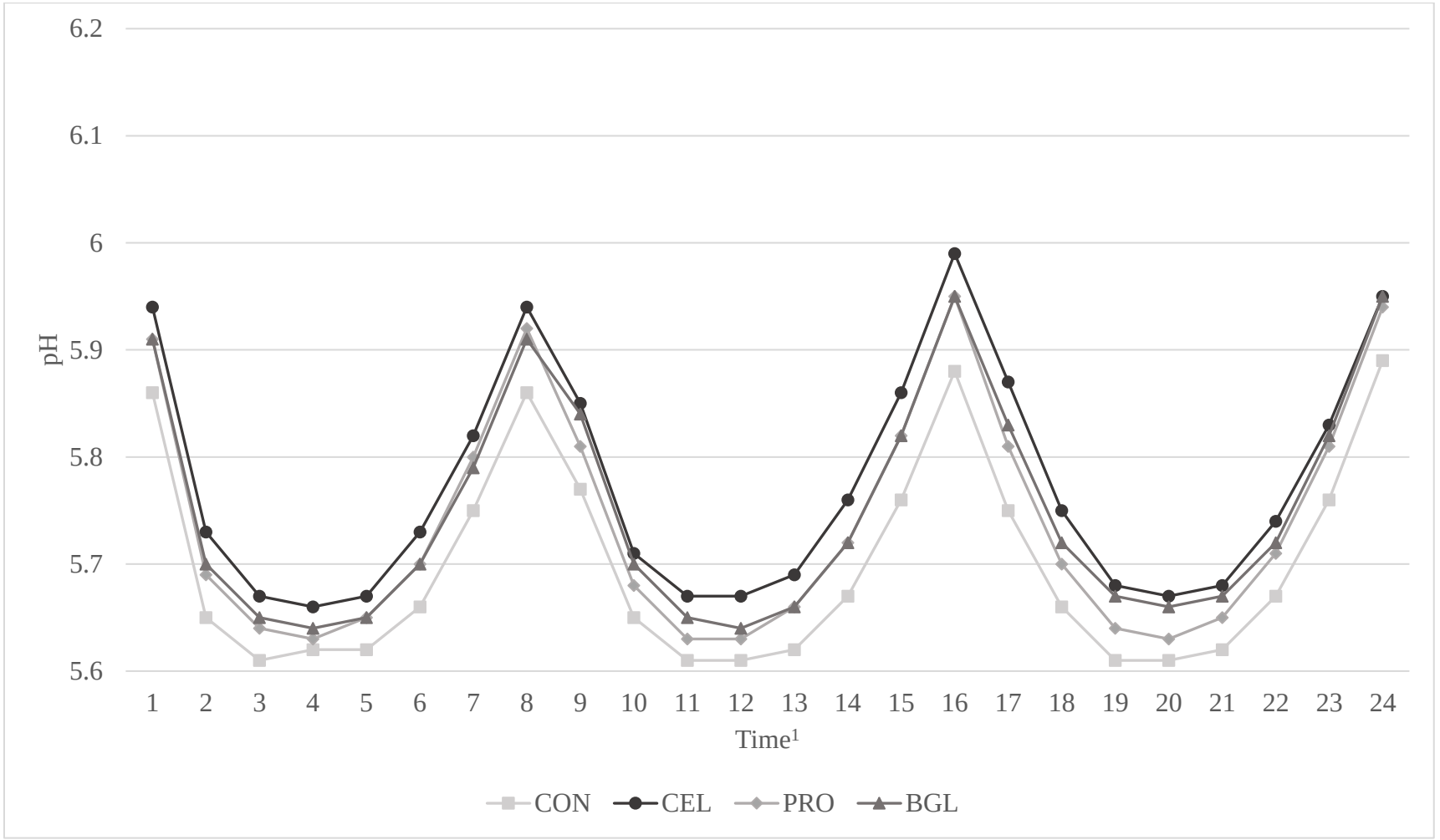
***** Signifies difference between least square means of enzyme and diet interaction, ($P < 0.05$)

Figure 8. Effect of enzyme supplementation on diets with varying forage:concentrate on PF in batch culture.



[a,b,c] Bars without a common superscript differ, $P < 0.05$.

Figure 9. Effects of exogenous enzyme supplementation on pH in continuous culture.



[1]Averaged over the 3 d sampling period

Bibliography

Adesogan, T., Z. X. Ma, J. J. Romero, and K. G. Arriola. 2014. Ruminant nutrition symposium: Improving cell wall digestion and animal performance with fibrolytic enzymes. J. Anim. Sci. 92:1317–1330. doi:10.2527/jas2013-7273.

Adler, J. H., J. A. Dye, D. E. Boggs, and H. H. Williams. 1958. Growth of rumen microorganisms in an in vitro continuous-flow system on a protein-free diet. Cornell Vet. 53–66.

Albersheim, P. 1975. The walls of growing plant cells. Sci. Am. 232:80–95.

AOAC. 1990. Official methods of analysis of the AOAC. 15th ed. Artlington, VA.

AOAC. 2005. Official methods of analysis of AOAC. 18th ed. AOAC International, Gaithersburg, MD.

AOCS. 2017. Official methods and recommended practices of the AOCS. 7th ed. Urbana, IL.

Arriola, K. G., A. S. Oliveira, Z. X. Ma, I. J. Lean, M. C. Giurcanu, and A. T. Adesogan. 2017. A meta-analysis on the effect of dietary application of exogenous fibrolytic enzymes on the performance of dairy cows. J. Dairy Sci. 100:4513–4527. doi:10.3168/jds.2016-12103.

Bacic, A., P. J. Harris, and B. A. Stone. 1988. Structure and function of plant cell walls. In: P. Stumpf and E. . Conn, editors. The biochemistry of plants. Academic Press. p. 297–371.

Balci, F., S. Dikmen, H. Gencoglu, A. Orman, I. I. Turkmen, and & H. Biricik. 2007. The effect of fibrolytic exogenous enzyme on fattening performance of steers. Bulg. J. Vet. Med. 10:113-118

Bannink, A., J. France, S. Lopez, W. J. J. Gerrits, E. Kebreab, S. Tamminga, and J. Dijkstra. 2008. Modelling the implication of feeding strategy on rumen fermentation and functioning of the rumen wall. Anim. Feed Sci. Technol. 143:3–26.

Bartolome, B., C. B. Faulds, M. Tuohy, G. P. Hazlewood, H. J. Gilbert, and G. Williamson. 1995. Influence of different xylanases on the activity of ferulic acid esterase on wheat bran. Biotechnol. Bioeng. 22:65–73.

Beauchemin, K. A., D. Colombatto, and D. P. Morgavi. 2004. A rationale for the development of feed enzyme products for ruminants. Can. J. Anim. Sci. 84:23–36.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:37–47.

Beauchemin, K. A., S. D. M. Jones, L. M. Rode, and V. J. H. Sewalt. 1997. Effects of fibrolytic enzymes in corn or barley diets on performance and carcass characteristics of feedlot cattle. Can. J. Anim. Sci. 77:645–653.

Beauchemin, K. A., L. M. Rode, and D. Karren. 1999a. Use of feed enzymes in feedlot finishing diets. Can. J. Anim. Sci. 79:243–246.

Beauchemin, K. A., L. M. Rode, and V. J. H. Sewaltl. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641–644.

Beauchemin, K. A., W. Z. Yang, and L. M. Rode. 1999b. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. J. Dairy Sci. 82:378–390. doi:10.3168/jds.S0022-0302(99)75244-6.

Beguin, P., and J.-P. Aubert. 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. 13:25–58. doi: 10.1111/j.1574-6976.1994.tb00033.x.

Bhat, M. K., and G. P. Hazlewood. 2001. Enzymology and other characteristics of cellulases and xylanases. In: M. Bedford and G. Partridges, editors. Enzymes in farm animal nutrition. CABI Publishing, Oxford. p. 11–60.

Biely, P. 1985. Microbial xylanolytic systems. Trends Biotechnol. 3:286–290.

Blummel, M., and P. Lebzien. 2001. Predicting ruminal microbial efficiencies of dairy rations by in vitro techniques. Livest. Prod. Sci. 68:107–117.

Blümmel, M., H. P. S. Makkar, and K. Becker. 1997. In vitro gas production: a technique revisited. J. Anim. Physiol. Anim. Nutr. (Berl). 77:24–34. doi:10.1111/j.1439-0396.1997.tb00734.x.

Bowman, G. R., K. A. Beauchemin, and J. A. Shelford. 2002. The Proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. J. Dairy Sci. 85:3420–3429. doi:10.3168/jds.S0022-0302(02)74430-5.

Burroughs, W., W. Woods, S. A. Ewing, J. Greig, and B. Theurer. 1960. Enzyme additions to fattening cattle rations. J. Anim. Sci. 19:458

Calsamiglia, S., A. Ferret, and M. Devant. 2002. Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. J. Dairy Sci. 85:574–579. doi:10.3168/jds.S0022-0302(02)74111-8.

Cheng, K.-J., C. W. Forsberg, H. Minato, and J. W. Corsterton. 1991. Microbial ecology and physiology of feed digestion within the rumen. In: T. Tsuda, Y. Sasaki, and R. Kawashina, editors. Physiological aspects of digestion and metabolism in ruminants. Academic Press, San Diego, CA. p. 595–624.

Clark, J., I. Dyer, and J. Templeton. 1961. Some nutritional and physiological effects of enzymes for fattening cattle. J. Anim. Sci. 20:928.

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy dows. J. Dairy Sci. 75:2304–2323. doi:10.3168/jds.S0022-0302(92)77992-2.

Cole, H. H., and M. Ronning. 1974. Animal agriculture. The biology of domestic animals and their use by man. WH Freeman and Co., San Francisco, CA.

Colombatto, D., G. Hervás, W. Z. Yang, and K. A. Beauchemin. 2003a. Effects of enzyme supplementation of a total mixed ration on microbial fermentation in continuous culture, maintained at high and low pH. J. Anim. Sci. 81:2617–2627. doi:10.2527/2003.81102617x.

Colombatto, D., D. P. Morgavi, A. F. Furtado, and K. A. Beauchemin. 2003b. Screening of exogenous enzymes for ruminant diets: Relationship between biochemical characteristics

and in vitro ruminal degradation1. J. Anim. Sci. 81:2628–2638. doi:10.2527/2003.81102628x.

Colombatto, D., F. L. Mould, M. K. Bhat, D. P. Morgavi, K. A. Beauchemin, and E. Owen. 2003c. Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms in vitro. J. Anim. Sci. 81:1040–1050.

Colombatto, D., F. L. Mould, M. K. Bhat, and E. Owen. 2007. Influence of exogenous fibrolytic enzyme level and incubation pH on the in vitro ruminal fermentation of alfalfa stems. Anim. Feed Sci. Technol. 137:150–162. doi:10.1016/j.anifeedsci.2006.10.001.

Considine, P., and M. Coughlan. 1989. Production of carbohydrate-hydolysing enzyme blends by solid-state fermentation. In: M. Coughlan, editor. Enzyme systems for lignocellulose degradation. Elsevier Science Publishers LTD, New York, NY. p. 273–282.

Dado, R. G., and M. S. Allen. 1995. Intake limitations, feeding Behavior, and rumen function of cows challenged with rumen fill from dietary fiber or inert bulk. J. Dairy Sci. 78:118–133. doi:10.3168/jds.S0022-0302(95)76622-X.

DeFrain, J. M., A. R. Hippen, K. F. Kalscheur, and J. M. Tricarico. 2005. Effects of dietary α-amylase on metabolism and performance of transition dairy cows. J. Dairy Sci. 88:4405–4413. doi:10.3168/JDS.S0022-0302(05)73127-1.

Dijkstra, J., J. L. Ellis, E. Kebreab, A. B. Strathe, S. López, J. France, and A. Bannink. 2012. Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Technol. 172:22–33. doi:10.1016/J.ANIFEEDSCI.2011.12.005.

Eadie, J. M., and P. N. Hobson. 1962. Effect of the Presence or Absence of Rumen Ciliate Protozoa on the Total Rumen Bacterial Count in Lambs. Nature. 193:503–505. doi:10.1038/193503a0.

Erfle, J. D., R. J. Boila, R. M. Teather, S. Mahadevan, and F. D. Sauer. 1982. Effect of pH on fermentation characteristics and protein degradation by rumen microorganisms in vitro. J. Dairy Sci. 65:1457–1464. doi:10.3168/jds.S0022-0302(82)82368-0.

Eun, J.-S., and K. A. Beauchemin. 2005. Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation, and milk production. J. Dairy Sci. 88:2140–2153. doi:10.3168/jds.S0022-0302(05)72890-3.

Eun, J.-S., and K. A. Beauchemin. 2007a. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using in vitro fermentation characteristics. Anim. Feed Sci. Technol. 132:298–315. doi:10.1016/j.anifeedsci.2006.02.014.

Eun, J.-S., and K. A. Beauchemin. 2007b. Enhancing in vitro degradation of alfalfa hay and corn silage using feed enzymes. J. Dairy Sci. 90:2839–2851. doi:10.3168/jds.2006-820.

Eun, J.-S., K. A. Beauchemin, S.-H. Hong, and M. W. Bauer. 2006. Exogenous enzymes added to untreated or ammoniated rice straw: Effects on in vitro fermentation characteristics and degradability. Anim. Feed Sci. Technol. 131:86–101. doi:10.1016/j.anifeedsci.2006.01.026.

Fan, L., Y. Lee, and D. Deardmore. 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng. 23:419–424.

Feng, P., C. W. Hunt, G. T. Pritchard, and W. E. Julien. 1996. Effect of enzyme preparations on in situ and in vitro degradation and in vivo digestive characteristics of mature cool-season grass forage in beef steers. J. Anim. Sci. 74:1349–1357.

Firkins, J. L., L. L. Berger, N. R. Merchen, G. C. Fahey, and D. R. Nelson. 1986. Effects of feed intake and protein degradability on ruminal characteristics and site of digestion in steers. J. Dairy Sci. 69:2111–2123. doi:10.3168/jds.S0022-0302(86)80643-9.

Firkins, J. L., M. L. Eastridge, N. R. St-Pierre, and S. M. Noftsger. 2001. Effects of grain variability and processing on starch utilization by lactating dairy cattle. J. Anim. Sci. 79:E218–E238.

Forsberg, C. W., L. K. A. Lovelock, L. Krumholz, and J. G. Buchanan-Smith. 1984. Protease activities of rumen protozoa. App. Env. Micr. 47:101-110

French, D. 1973. Chemical and physical properties of starch. J. Anim. Sci. 37:1048–1061. doi:https://doi.org/10.2527/jas1973.3741048x.

Fries, G. F., C. A. Lasslter, and C. F. Huffman. 1958. The effect of enzyme supplementation of milk replacers on the growth rate of calves. J. Dairy Sci. 41:1081–1087.

Gado, H. M., A. Z. M. Salem, P. H. Robinson, and M. Hassan. 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. Anim. Feed Sci. Technol. 154:36–46. doi:10.1016/j.anifeedsci.2009.07.006.

Giraldo, L., M. Tejido, and M. Ranilla. 2008. Effects of exogenous fibrolytic enzymes on in vitro ruminal fermentation of substrates with different forage:concentrate ratios. Anim. Feed Sci. Technol. 141:306–325. doi:10.1016/j.anifeedsci.2007.06.013.

Goering, H., and Van Soest. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington D.C.

Gómez-Vázquez, A., G. D. Mendoza, E. Aranda, J. Pérez, A. Hernández, and J. M. Pinos-Rodríguez. 2011. Influence of fibrolytic enzymes on growth performance and digestion in steers grazing stargrass and supplemented with fermented sugarcane. J. Appl. Anim. Res. 39:77–79. doi:10.1080/09712119.2011.558670.

Hannah, S. M., M. D. Stern, and F. R. Ehle. 1986. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation in vivo of mixed diets containing various soya bean products. Anim. Feed Sci. Technol. 16:51-62

Herrera-Saldana, R. E., J. . Huber, and M. H. Poore. 1990. Dry matter, crude protein, and starch degradability of five cereal grains. J. Dairy Sci. 73:2386–2393. doi:10.3168/JDS.S0022-0302(90)78922-9.

Holtshausen, L., Y.-H. Chung, H. Gerardo-Cuervo, M. Oba, and K. A. Beauchemin. 2011. Improved milk production efficiency in early lactation dairy cattle with dietary addition

of a developmental fibrolytic enzyme additive. J. Dairy Sci. 94:899–907. doi:10.3168/jds.2010-3573.

Hoover, W. H., B. A. Crooker, and C. J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continous cultures of rumen contents. J. Anim. Sci. 43:528–534. doi:10.2527/jas1976.432528x.

Hristov, A. N., C. E. Basel, A. Melgar, A. E. Foley, J. K. Ropp, C. W. Hunt, and J. M. Tricarico. 2008. Effect of exogenous polysaccharide-degrading enzyme preparations on ruminal fermentation and digestibility of nutrients in dairy cows. Anim. Feed Sci. Technol. 145:182–193. doi:10.1016/j.anifeedsci.2007.05.051.

Hristov, A. N., C. Lee, R. Hristova, P. Huhtanen, and J. L. Firkins. 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. J. Dairy Sci. 95:5299–5307. doi:10.3168/JDS.2012-5533.

Hristov, A. N., T. A. Mcallister, and K.-J. Cheng. 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: Effects on nutrient digestion in cattle fed a barley grain diet. J. Anim. Sci. 78:477–487.

Huang, Z., P. Urriola, I. Salfer, M. Stern, and G. Shurson. 2017. Differences in in vitro hydrolysis and fermentation among and within high-fiber ingredients using a modified three-step procedure in growing pigs. J. Anim. Sci. 95:5497–5506. doi:10.2527/jas2017.1907.

Huntington, G. B. 1997. Starch utilization by ruminants: From basics to the bunk. J. Anim. Sci. 75:852–867.

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492. doi:10.2527/1995.7382483x.

Jung, H. G., and M. S. Allen. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73:2774–90.

Klingerman, C. M., W. Hu, E. E. McDonell, M. C. DerBedrosian, and L. Kung. 2009. An evaluation of exogenous enzymes with amylolytic activity for dairy cows. J. Dairy Sci. 92:1050–1059. doi:10.3168/jds.2008-1339.

Kotarski, S. F., R. D. Waniska, and K. K. Thurn. 1992. Starch hydrolysis by the ruminal microflora. J. Nutr. 122:178–190. doi:10.1093/jn/122.1.178.

Krueger, N. A., and A. T. Adesogan. 2008. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. Anim. Feed Sci. Technol. 145:84–94. doi:10.1016/j.anifeedsci.2007.05.041.

Krueger, N. A., A. T. Adesogan, C. R. Staples, W. K. Krueger, S. C. Kim, R. C. Littell, and L. E. Sollenberger. 2008. Effect of method of applying fibrolytic enzymes or ammonia to Bermudagrass hay on feed intake, digestion, and growth of beef steers 1. J. Anim. Sci. 86:882–889. doi:10.2527/jas.2006-717.

Kumar, S., S. Singh Dagar, S. Kumar Sirohi, R. Chandra Upadhyay, and A. Kumar Puniya. 2013. Microbial profiles, in vitro gas production and dry matter digestibility based on various

ratios of roughage to concentrate. Ann. Microbiol. 63:541–545. doi:10.1007/s13213-012-0501-0.

Lazaridou, A., and C. G. Biliaderis. 2007. Molecular aspects of cereal b-glucan functionality: Physical properties, technological applications and physiological effects. J. Cereal Sci. 46:101–118. doi:10.1016/j.jcs.2007.05.003.

Lee, B., A. L. Pometto, A. Demirci, and P. N. Hinz. 1998. Media evaluation for the production of microbial enzymes. J. Agri. 46:4775–4778. doi:10.1021/jf980476q.

Lopuszanka-Rusek, M., and K. Bilik. 2011. Influence of pre-and postpartum supplementation of fibrolytic enzymes and yeast culture, or both, on performance and metabolic status of dairy cows. Ann. Anim. Sci. 11:531–545. doi:10.2478/v10220-011-0005-z.

Mcallister, T. A., S. J. Oosting, J. D. Popp, Z. Mir, L. J. Yanke, A. N. Hristov, R. J. Treacher, K.-J. Cheng, Z. Yanke, L. J. Hristov, R. J. Cheng, and R. J. Et Cheng. 1999. Effect of exogenous enzymes on digestibility of barley silage and growth performance of feedlot cattle. Can. J. Anim. Sci. 79:353–360.

McAllister, T. A., R. C. Phillippe, L. M. Rode, and K.-J. Cheng. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. J. Anim. Sci. 71:205–212. doi:10.2527/1993.711205x.

Meale, S. J., K. A. Beauchemin, A. N. Hristov, A. V Chaves, and T. A. Mcallister. 2014. Opportunities and challenges in using exogenous enzymes to improve ruminant production. J. Anim. Sci. 92:427–442. doi:10.2527/jas2013-6869.

Menke, K. H., and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28:7-55

Morgavi, D. P., K. A. Beauchemin, V. L. Nsereko, L. M. Rode, T. A. McAllister, A. D. Iwaasa, Y. Wang, and W. Z. Yang. 2001. Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. J. Anim. Sci. 79:1621–1630. doi:10.2527/2001.7961621x.

Nazmín Tirado-González, D., L. Alberto Miranda-Romero, A. Ruíz-Flores, S. Ernesto Medina-Cuéllar, R. Ramírez-Valverde, and G. Tirado-Estrada. 2018. Meta-analysis: effects of exogenous fibrolytic enzymes in ruminant diets. J. Appl. Anim. Res. 46:771–783. doi:10.1080/09712119.2017.1399135.

Nelson, F., and V. Damon. 1960. Comparison of different supplemental enzymes with and without diethylstilbestrol for fattening steers. J. Anim. Sci. 19:1279.

Netke, S. P., K. E. Gardner, and E. A. Kendall. 1960. Physiological responses of dairy calves to certain carbohydrates when added to a milk replacer diet. J. Dairy Sci. 43:892.

Nsereko, V. L., D. P. Morgavi, L. M. Rode, K. A. Beauchemin, and T. A. Mcallister. 2000. Effects of fungal enzyme preparations on hydrolysis and subsequent degradation of alfalfa hay by mixed rumen microorganisms in vitro. Anim. Feed Sci. Technol. 88:153–170.

Oba, M., and M. S. Allen. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. J. Dairy Sci. 82:589–596. doi:10.3168/jds.S0022-0302(99)75271-9.

Oba, M., and M. S. Allen. 2000. Effects of brown midrib 3 mutation in corn silage on productivitiy of dairy cows fed two concentration of dietarty neutral detergent fiber: Digestibility and microbial efficiency. J. Dairy Sci. 83:1350–1358.

Okamota, M. J., V. R. Thomas, and T. L. Johnson. 1959. Utilization of various carbohydrates by young calves. J. Dairy Sci. 42:920 (Abstr.).

Oltjen, J. W., and J. L. Beckett. 1996. Role of ruminant livestock in sustainable agricultural systems. J. Anim. Sci. 74:1406–1409.

Ortiz-Rodea, A., A. Noriega-Carrillo, A. Z. M. Salem, O. C. Ortega, and M. González-Ronquillo. 2013. The use of exogenous enzymes in dairy cattle on milk production and their chemical composition: A meta-analysis. Anim. Nutr. Feed Technol. 13:399–409.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1996. Acidosis in cattle: a review. J. Anim. Sci. 76:275–286.

Oxford, A. E. 1955. The bacteriology and protozoology of ruminant digestion. J. Sci. Food Agric. 6:413–418. doi:10.1002/jsfa.2740060801.

Perry, T., W. Beeson, and D. Cope. 1960. Low vs high shelled corn with and without enzymes and stillbestrol for fattening steers. J. Anim. Sci. 19:1284

Peters, A., U. Meyer, and S. Dänicke. 2015. Effect of exogenous fibrolytic enzymes on performance and blood profile in early and mid-lactation Holstein cows. Anim. Nutr. 1:229–238. doi:10.1016/J.ANINU.2015.09.001.

Phakachoed, N., W. Suksombat, D. Colombatto, and K. A. Beauchemin. 2013. Use of fibrolytic enzymes additives to enhance in vitro ruminal fermentation of corn silage. Livest. Sci. 157:100–112. doi:10.1016/j.livsci.2013.06.020.

Prins, R. A., D. L. Van Rheenen, A. T. Van, T. Klooster, and V. Rheenen. 1983. Characterization of microbial proteolytic enzymes in the rumen. Antonie Van Leeuwenhoek. 49:585–595.

Ralph, J., K. Lundquist, G. Brunow, F. Lu, H. Kim, P. F. Schatz, J. M. Marita, R. D. Hatfield, S. A. Ralph, J. H. Christensen, and W. Boerjan. 2004. Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids. Phytochem. Rev. 3:26-60

Ravindran, R., and A. K. Jaiswal. 2016. Microbial enzyme production using lignocellulosic food industry wastes as feedstock: A review. Bioengineering. 3:1–22. doi:10.3390/bioengineering3040030.

Rode, L. M., W. Z. Yang, and K. A. Beauchemin. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. J. Dairy Sci. 82:2121–2126. doi:10.3168/jds.S0022-0302(99)75455-X.

Rojo Rubio, M., M. Martínez, C. Galván, and M. Magdalena. 2001. Use of thermostable amylase from Bacillus licheniformis on in vitro starch digestion of sorghum and corn. Agroceiencia. 35:423-427

Romero, J. J., E. G. Macias, Z. X. Ma, R. M. Martins, C. R. Staples, K. A. Beauchemin, and A. T. Adesogan. 2016. Improving the performance of dairy cattle with a xylanase-rich exogenous enzyme preparation. J. Dairy Sci. 99:3486–3496. doi:10.3168/JDS.2015-10082.

Rooney, L. W., and R. L. Pflugfelder. 1986. Factors affecting starch digestibility with special emphasis on sorghum and corn. J. Anim. Sci. 63:1607–23.

Rovics and Ely. 1962. Response of beef cattle to enzyme supplements. J. Anim. Sci. 21:1012. doi:10.2527/jas1962.214969x.

Russell, J. B., and D. B. Wilson. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? J. Dairy Sci. 79:1503–1509. doi:10.3168/JDS.S0022-0302(96)76510-4.

Savelkoul, H. F. J., W. Chanput, and H. J. Wichers. 2013. Immunomodulatory effects of mushroom β-glucans. In: P. Calder, P. Yaqoob, editors, Diet, Immunity and Inflammation. Woodhead Publishing, Cambridge, UK. p. 416–434.

Scheller, H. V., and P. Ulvskov. 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61:263–289. doi:10.1146/annurev-arplant-042809-112315.

Selinger, L. B., C. W. Forsberg, and K.-J. Cheng. 1996. The rumen: A unique source of enzymes for enhancing livestock production. Anaerobe. 2:263-284

Siciliano-Jones, J., and M. R. Murphy. 1988. Production of volatile fatty acids in the rumen and cecum-colon of steers as affected by forage:concentrate and forage physical form. J. Dairy Sci. 72:485–492. doi:10.3168/jds.S0022-0302(89)79130-X.

Slyter, L. L., W. Nelson, and M. J. Wolin. 1964. Modifications of a device for maintenance of the rumen microbial population in continuous culture. App. Microbiol. 12:374-377

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. Comstock Pub. Ithaca, NY

Stewart, D. G., R. G. Warner, and H. W. Seeley. 1961. Continuous culture as a method for studying rumen fermentation. Appl. Microbiol. 9:150–156.

Sujani, S., and R. T. Seresinhe. 2015. Exogenous enzymes in ruminant nutrition: A review. Asian J. Anim. Sci. 9:85–99. doi:10.3923/ajas.2015.85.99.

Tenkanen, M., M. Vrsanska, M. Siika-aho, D. W. Wong, V. Puchart, M. Penttila, M. Saloheimo, and P. Biely. 2013. Xylanase XYN IV from Trichoderma reesei showing exo-and endo-xylanase activity. FEBS J. 280:285–301. doi:10.1111/febs.12069.

Tilley, J. M. A., and R. A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. Grass Forage Sci. 18:104–111. doi:10.1111/j.1365-2494.1963.tb00335.x.

Tricarico, J. M., M. D. Abney, M. L. Galyean, J. D. Rivera, K. C. Hanson, K. R. McLeod, and D. L. Harmon. 2007. Effects of a dietary Aspergillus oryzae extract containing α-amylase activity on performance and carcass characteristics of finishing beef cattle. J. Anim. Sci. 85:802–811. doi:10.2527/jas.2006-427.

Tricarico, J. M., and K. A. Dawson. 2005. Influence of supplemental endoglucanase or xylanase on volatile fatty acid production from ruminant feed by ruminal in vitro cultures. Arch. Anim. Nutr. 59:325–334. doi:10.1080/17450390500247865.

Vargas, J. M., G. D. Mendoza, M. De la Salud Rubio-Lozano, and F. A. Castrjon. 2013. Effect of exogenous fibrolytic enzymes on the carcass characteristics and performance of grain-finished steers. Anim. Nutr. Feed Technol. 13:435–439.

Vera, J., A. Smith, D. R. ZoBell, A. J. Young, and J.-S. Eun. 2012. Effects of an exogenous proteolytic enzyme on growth performance of beef steers and in vitro ruminal fermentation in continuous culture. Prof. Anim. Sci. 28:452–463.

Wallace, R. 1996. The proteolytic systems of ruminal microorganisms. Ann. Zootech. 45:301–308.

Wallace, R. J., and T. L. Brammall. 1985. The role of different species of bacteria in the hydrolysis of protein in the rumen. J. Gen. Microbiol. 131:821-832

Van Der Walt, J. G., and J. H. F. Meyer. 1988. Protein digestion in ruminants. S. Afr. J. Anim. Sci. 18:30–41.

Weiss, W. P., and W. L. Shockey. 1991. Value of orchardgrass and alfalfa silages fed with varying amounts of concentrates to dairy cows,. J. Dairy Sci. 74:1933–1943. doi:10.3168/JDS.S0022-0302(91)78359-8.

Weller, R. A., and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rument of the sheep and from a continuous in vitro fermentation system. Br. J. Nutr. 32:341–351. doi:10.1079/BJN19740087.

White, B. A., R. I. Mackie, and K. . Doerner. 1993. Enaymatic hydrolysis of forage cell walls. In: H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, editors. Forage cell wall struture and digestibility. ASA, CSSA, SSSA, Madison, WI. p. 455–498.

Williams, J., and C. Knodt. 1951. The suppplementation of milk replacements with enzymes and other products. J. Anim. Sci. 10:975–980.

Yang, W. Z., K. A. Beauchemin, and L. M. Rode. 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. J. Dairy Sci. 82:391–403. doi:10.3168/JDS.S0022-0302(99)75245-8.

Yang, W. Z., K. A. Beauchemin, and L. M. Rode. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. J. Dairy Sci. 83:2512–2520. doi:10.3168/jds.S0022-0302(00)75143-5.

Yang, W. Z., K. A. Beauchemin, and D. D. Vedres. 2002. Effects of pH and fibrolytic enzymes on digestibility, bacterial protein synthesis, and fermentation in continuous culture. Anim. Feed. Sci. Tech. 102:137-150.

Young, K., J. Lim, M. Der Bedrosian, and L. Kung Jr. 2012. Effect of exogenous protease enzymes on the fermentation and nutritive value of corn silage. J. Dairy Sci. 95:6687–6694. doi:10.3168/jds.2012-5628.

Yu, P., J. J. Mckinnon, and D. A. Christensen. 2005. Improving the nutritional value of oat hulls for ruminant animals with pretreatment of a multienzyme cocktail: In vitro studies. J. Anim. Sci. 83:1133–1141.

Zinn, R. A., and F. N. Owens. 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. 66:157–166.